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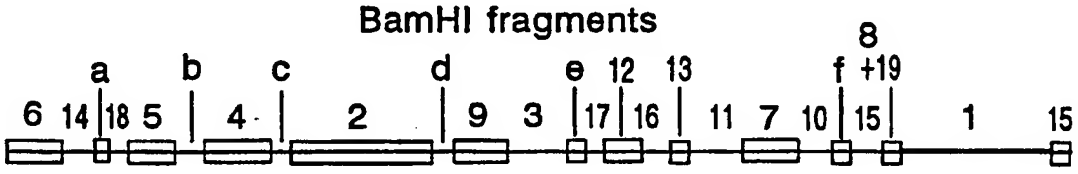

(57) Abstract

The present invention provides a recombinant herpesvirus of turkeys comprising a foreign gene inserted within the genomic DNA of herpesvirus of turkeys, wherein the foreign gene is inserted into the unique *Stu*I site in the US2 gene coding region of the herpesvirus of turkeys genomic DNA and is capable of being expressed in a host cell infected with herpesvirus of turkeys. In one embodiment of the invention, said foreign gene encodes a polypeptide which is antigenic in an animal into which the recombinant herpesvirus of turkeys is introduced. The present invention also relates to the following recombinant herpesvirus of turkeys constructs and vaccines containing the same: S-HVT-012, S-HVT-045, S-HVT-046, S-HVT-047, S-HVT-062, S-HVT-048, S-HVT-049, S-HVT-050, S-HVT-106, S-HVT-051, S-HVT-052, S-HVT-066 and S-HVT-096.

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RECOMBINANT HERPESVIRUS OF TURKEYS AND USES THEREOF

Background Of The Invention

The ability to isolate DNA and clone such isolated DNA into bacterial plasmids has greatly expanded the approaches available to make viral vaccines. The methods used to make the present invention involve modifying cloned DNA sequences from various pathogens of animals, by insertions, deletions, single or multiple base changes, and subsequent insertions of these modified sequences into the genome of the virus. One utility of the addition of foreign sequences is achieved when the foreign sequence encodes a foreign protein that is expressed during viral infection of the animal. The resulting live virus may then be used in a vaccine to elicit an immune response in a host animal and provide protection to the animal against disease. A virus with these characteristics is referred to as a viral vector, because it becomes a living vector that will carry and express the foreign protein in the host animal. In effect it becomes an elaborate delivery system for the foreign protein(s).

More specifically, the present invention relates to the use of herpesvirus of turkeys (HVT) as a viral vector for vaccination of birds against disease. The group of herpesviruses comprise various pathogenic agents that infect and cause disease in a number of target species: swine, cattle, chickens, horses, dogs, cats, etc. Each herpesvirus is specific for its host species, but they are all related in the structure of their genomes, their mode of

replication, and to some extent in the pathology they cause in the host animal and in the mechanism of the host immune response to the virus infection.

The application of recombinant DNA techniques to animal viruses has a relatively recent history. The first viruses to be engineered have been those with the smallest genomes. In the case of the papovaviruses, because these viruses are so small and cannot accommodate much extra DNA, their use in genetic engineering has been as defective replicons. Foreign gene expression from these viruses requires a wild-type helper virus and is limited to cell culture systems. For adenoviruses, there is a small amount of nonessential DNA that can be replaced by foreign sequences. The only foreign DNA that seems to have been expressed in adenoviruses are the T-antigen genes from papovaviruses (Mansour, *et al., Proc. Natl. Acad. Sci. US*, 1985; Thummel, *et al., Cell*, 1983; Scolnick, *et al., Cell*, 1981; Thummel, *et al., Cell*, 1981), and the herpes simplex virus (HSV) thymidine kinase gene (Haj-Ahmed and Graham, *J. of Virology*, 1986). These publications do not identify the nonessential regions in HVT wherein foreign DNA may be inserted, nor do they teach how to achieve the expression of foreign genes in HVT, e.g., which promoter sequence and termination sequence to use.

Another group of viruses that have been engineered are the poxviruses. One member of this group, vaccinia, has been the subject of much research on foreign gene expression. Poxviruses are large DNA-containing viruses that replicate in the cytoplasm of the infected cell. They have a structure that is unique in that they do not contain any capsid that is based upon icosahedral symmetry or helical symmetry. The poxviruses are most likely to have evolved

from bacterial-like microorganisms through the loss of function and degeneration. In part due to this uniqueness, the advances made in the genetic engineering of poxviruses cannot be directly extrapolated to other viral systems, including herpesviruses and HVT. Vaccinia recombinant virus constructs have been made in a number of laboratories that express the following inserted foreign genes: HSV thymidine kinase gene (Mackett, *et al., Proc. Natl. Acad. Sci.* USA, 1982; Panicali and Paoletti, *Proc. Natl. Acad. Sci.* USA, 1982, hepatitis B surface antigen (Paoletti, *et al., Proc. Natl. Acad. Sci. USA*, 1984; Smith et al., *Nature*, 1983), HSV glycoprotein D gene, influenzae hemagglutinin gene (Panicali, *et al., Proc. Natl. Acad. Sci. USA*, 1983; Smith, *et al., Proc. Natl. Acad. Sci. USA*, 1983), malaria antigen gene (Smith, *et al., Science*, 1984, and vesicular stomatitis glycoprotein G gent (Mackett, *et al., Science*, 1986). The general overall features of vaccinia recombinant DNA work are similar to the techniques used for all the viruses, especially as they relate to the techniques in reference (Maniatis, *et al., Molecular Cloning*, 1982). However in detail, the vaccinia techniques are not applicable to herpesviruses and HVT. The utility of vaccinia as a vaccine vector is in question because of its close relationship to human smallpox and its known pathogenicity to humans. Thus, the use of the host-specific herpesvirus HVT is a better solution to vaccination of poultry.

Among the primate herpesviruses, only HSV of humans and, to a limited extent, herpes saimiri of monkeys have been engineered to contain foreign DNA sequences. The first use of recombinant DNA to manipulate HSV involved cloning a piece of DNA from the L-S junction region into the unique long region of HSV DNA, specifically into the thymidine kinase gene (Moccarski, *et al., Cell*, 1980). This insert was not a foreign piece of DNA, rather it was a naturally

occurring piece of herpesvirus DNA that was duplicated at another place in the genome. This piece of DNA was not engineered to specifically express a protein, and thus this work does not involve expression of protein in herpesviruses. The next manipulation of HSV involved the creation of deletions in the virus genome by a combination of recombinant DNA techniques and thymidine kinase selection. Using this approach, the HSV alpha-22 gene has been deleted (Post, *et al.*, *Cell*, 1981), and a 15,000 basepair sequence of DNA has been deleted from the internal repeat of HSV (Poffenberger, *et al.*, *Proc. Natl. Acad. Sci. USA*, 1981).

The following cases involve insertion of genes that encode protein into herpesviruses: the insertion of HSV glycoprotein C into a naturally occurring deletion mutant of this gene in HSV (Gibson and Spear, *J. of Virology*, 1983); the insertion of glycoprotein D of HSV type 2 into HSV type 1 (Lee, *et al.*, *Proc. Natl. Acad. Sci. USA*, 1982), with no manipulation of promoter sequences since the gene is not 'foreign'; the insertion of hepatitis B surface antigen into HSV under the control of the HSV ICP4 promoter (Shih, *et al.*, *Proc. Natl. Acad. Sci. USA*, 1984); and the insertion of bovine growth hormone into herpes saimiri virus with an SV40 promoter (the promoter did not work this system and an endogenous upstream served to transcribe the gene) (Desrosiers, *et al.*, 1984). Two additional foreign genes (chicken ovalbumin gene and Epstein-Barr virus nuclear antigen) have been inserted into HSV (Arsenakis and Roizman, 1984), and glycoprotein X of pseudorabies virus has been inserted into HSV (Post, et al., 1985).

These cases of deletion or insertion of genes into herpesviruses demonstrate that it is possible to genetically engineer herpesvirus genomes by recombinant DNA techniques. The methods that have been used to insert genes involve homologous recombination between the viral DNA cloned in plasmids and purified viral DNA transfected into the same animal cell. However, the extent to which one can generalize the location of the deletion and the sites for insertion of foreign genes is not known from these previous studies.

One object of the present invention is a vaccine for Marek's disease. Marek's disease virus (MDV) is the causative agent of Marek's disease which encompasses fowl paralysis, a common lymphoproliferative disease of chickens. The disease occurs most commonly in young chickens between 2 and 5 months of age. The prominent clinical signs are progressive paralysis of one or more of the extremities, incoordination due to paralysis of legs, drooping of the limb due to wing involvement, and a lowered head position due to involvement of the neck muscles. In acute cases, severe depression may result. In the case of highly oncogenic strains, there is characteristic bursal and thymic atrophy. In addition, there are lymphoid tumors affecting the gonads, lungs, liver, spleen, kidney and thymus (Mohanty and Dutta, 1981).

Most chickens are vaccinated against MDV at one day of age to protect the bird against MDV for life. Prior to the present invention, the principal vaccination method for MDV involved using naturally occurring strains of turkey herpesvirus (HVT). It would be advantageous to incorporate other antigens into this vaccination at one day of age, but efforts to combine conventional vaccines have not proven satisfactory to date due to competition and

immunosuppression between pathogens. The multivalent HVT-based vaccines engineered in this invention represent a novel way to simultaneously vaccinate against a number of different pathogens. For the first time, a recombinant HVT with a foreign gene inserted into a non-essential region of the HVT genome is disclosed.

The types of genetic engineering that have been performed on these herpesviruses consist of cloning parts of the virus DNA into plasmids in bacteria, reconstructuring the virus DNA while in the cloned state so that the DNA contains deletions of certain sequences, and furthermore adding foreign DNA sequences either in place of the deletions or at sites removed from the deletions.

A foreign gene of interest targeted for insertion into the genome of HVT may be obtained from any pathogenic organism of interest. Typically, the gene of interest will be derived from pathogens that in poultry cause diseases that have an economic impact on the poultry industry. The genes may be derived from organisms for which there are existing vaccines, and because of the novel advantages of the vectoring technology the HVT derived vaccines will be superior. Also, the gene of interest may be derived from pathogens for which there is currently no vaccine but where there is a requirement for control of the disease. Typically, the gene of interest encodes immunogenic polypeptides of the pathogen, and may represent surface proteins, secreted proteins and structural proteins.

A relevant avian pathogen that is a target for HVT vectoring is Infectious Laryngotracheitis virus (ILT). ILT is a member of the herpesviridiae family, and this pathogen causes an acute disease of chickens which is characterized by respiratory depression, gasping and expectoration of

bloody exudate. Viral replication is limited to cells of the respiratory tract, where in the trachea the infection gives rise to tissue erosion and hemorrhage. In chickens, no drug has been effective in reducing the degree of lesion formation or in decreasing clinical signs. Vaccination of birds with various modified forms of the ILT virus derived by cell passage and/or tedious regimes of administration have conferred acceptable protection in susceptible chickens. Because of the degree of attenuation of current ILT vaccines care must be taken to assure that the correct level of virus is maintained; enough to provide protection, but not enough to cause disease in the flock.

An additional target for the HVT vectoring approach is Newcastle disease, an infectious, highly contagious and debilitating disease that is caused by the Newcastle disease virus (NDV). NDV is a single-stranded RNA virus of the paramyxovirus family. The various pathotypes of NDV (velogenic, mesogenic, lentogenic) differ with regard to the severity of the disease, the specificity and symptoms, but most types seem to infect the respiratory system and the nervous system. NDV primarily infects chickens, turkeys and other avian species. Historically vaccination has been used to prevent disease, but because of maternal antibody interferences, life-span of the bird and route of administration, the producer needs to adapt immunization protocols to fit specific needs.

Summary Of The Invention

The present invention provides a recombinant herpesvirus of turkeys comprising a foreign gene inserted within the genomic DNA of herpesvirus of turkeys, wherein the foreign gene is inserted into the unique *Stu*I site in the US2 gene coding region of the herpesvirus of turkeys genomic DNA and is capable of being expressed in a host cell infected with herpesvirus of turkeys. In one embodiment of the invention, said foreign gene encodes a polypeptide which is antigenic in an animal into which the recombinant herpesvirus of turkeys is introduced.

The present invention also relates to the following recombinant herpesvirus of turkeys constructs and vaccines containing the same: S-HVT-012, S-HVT-045, S-HVT-046, S-HVT-047, S-HVT-062, S-HVT-048, S-HVT-049, S-HVT-050, S-HVT-106, S-HVT-051, S-HVT-052, S-HVT-066 and S-HVT-096.

Brief Description Of Figures

Fig. 1 shows details of HVT Construction and Map Data.

Figure IA shows *Bam*HI restriction fragment map of HVT. Fragments are numbered in order of decreasing size; letters refer to small fragments whose comparative size has not been determined.

Figure 1B shows *Bam*HI #16 fragment showing location of β-galactosidase gene insertion in S-HVT-001.

Figure 1C shows *Bam*HI #19 fragment showing location of β-galactosidase gene insertion.

Legend: B = *Bam*HI; X = *Xho*I; H = *Hind*III; P = *Pst*I; S = *Sal*I; N = *Nde*I; R = *Eco*RI.

Figure 2 (made up of figures 2A, 2B, 2C and 3D) shows insertion in Plasmid 191-47

Figure 3 shows details of S-HVT-003 Construction.

Figure 3A shows restriction map of HVT DNA in the region of the BamHI #16 fragment. This fragment is contained within large *Hind*III fragment. Figure 3A also shows the XhoI site which was first changed to an *Eco*RI (R) site by use of a "linker" and standard cloning procedures. Figure 3A also shows details of the construction of the beta-gal gene and IBVD gene inserted into the *BamHI* #16 fragment for use in homologous recombination. Both genes were under the control of the PRV gX gene promoter (gX).

Figure 3B show the S-HVT-003 genome, including the

location of the two inserted foreign genes, β-gal and IBDV.

In figure 3 : H = *Hind*III; B = *Bam*HI; X = *Xho*I; R = *Eco*RI; Xb = *Xba*I; Hp = *Hpa*I; S = *Sma*I; UL = unique long region; US = unique short region; IR = internal repeat region; TR = terminal repeat region.

Figure 4 shows a Western blot indicating the differential expression of the IBDV 32kD antigen in cellular lysates of S-HVT-003 infected cells (32kD present) and S-HVT-001 infected cells (32kD negative). IBDV specific polypeptides were identified by probing the blot with hyper-immune rat antiserum directed against denatured IBDV virions. This serum reacts primarily with the immunodominant 32kD antigen (VP3). The lanes on the blot contain: 1) protein molecular weight standards, 2) uninfected CEF cells, 3) S-HVT-001 infected CEF's, 4) 5) & 6) S-HVT-003 and 7) IBDV virion polypeptides.

Figure 5 shows a Western blot indicating the presence of the 42kD IBDV polypeptide (VP2) product in cellular lysates derived from CEF cells infected with either wild-type HVT virus or with S-HVT-001 that expresses β-galactosidase. IBDV specific polypeptides were identified using a VP-2 specific rabbit anti-peptide antiserum. The lanes contain: 1) protein molecular weight standards, 2) wild-type HVT infected CEF's, 3) S-HVT-001 infected CEF's, 4) S-HVT-003 infected CEF's, 5) S-HVT-003 infected CEF's, and 6) IBDV virion polypeptides.

Figure 6 provides details of S-HVT-004 Construction.

Figure 6A is a restriction map of HVT DNA in the region of the *Bam*HI #16 fragment. This fragment is contained

within a large *Hind*III fragment which was first changed to an *Eco*RI (R) site by use of a "linker" and standard cloning procedures.

Figure 6B provides details of the construction of the β-gal gene and MDV gA gene inserted into the *Bam*HI #16 fragment for use in homologous recombination. Beta-gal was under the control of the PRV gX gene promoter (gX), while the MDV gA gene was under the control of its own promoter.

Figure 6C is of S-HVT-004 genome showing the location of the two inserted foreign genes, β-gal and MDV gA.

In Figure 6, H = *Hind*III; B = *Bam*HI; X = *Xho*I; R = *Eco*RI; Xb = *Xba*I; UL = unique long region; US = unique short region; IR = internal repeat region; TR = terminal repeat region.

Figure 7 (made up of figures 7A and 7B) is a detailed description of the β-galactosidase (*lac*Z) marker gene insertion in homology vector 467-22.A12. The diagram shows the orientation of DNA fragments assembled in the marker gene. The origin of each fragment is described in the Materials and Methods section. The sequences located at the junctions between each fragment and at the ends of the marker gene are shown. The restriction sites used to generate each fragment are indicated at the appropriate junction. The location of the lacZ gene coding region is also given. Numbers in parenthesis () refer to amino acids, and restriction sites in brackets [] indicate the remnants of sites which were destroyed during construction. The following abbreviations are used, pseudorabies virus (PRV), lactose operon Z gene (lacZ), *Escherichia coli* (E.Coli), poly adenylation signal (pA), and glycoprotein X (gpX).

Figure 8 is a *Bam*HI, *Not*I restriction map of the HVT genome. The unique long UL and unique short US regions are shown. The long and short region repeats are indicated by boxes. The *Bam*HI fragments are numbered in decreasing order of size. The location of probes P1-P4 are indicated. The origin of each probe is as follows: P1 - *Bam*HI #6, P2 - *Bam*HI #2, P3 - *Bam*HI #13, and P4 - 4.0 kb *Bg*III to *Stu*I sub-fragment of HVT genomic *Xba*I fragment #5 (8.0 kb).

Figure 9 outlines the procedure for construction of plasmid pSY229.

Figure 10 (made up of figures 10A and 10B) is a detailed description of the MDV gene cassette insert in Homology Vectors 456-18.18 and 456-17.22. The diagram shows the orientation of DNA fragments assembled in the cassette. The origin of each fragment is described in the Materials and Methods section. The sequences located at the junctions between each fragment and at the ends of the marker gene are shown, including junction A (SEQ ID NO: 34), junction B (SEQ ID NO: 35), and junction C (SEQ ID NO: 36). The restriction sites used to generate each fragment are indicated at the appropriate junction. The location of the MDV gA and gB genes is also given. Numbers in parenthesis () refer to amino acids, and restriction sites in brackets [] indicate the remnants of sites which were destroyed during construction.

Figure 11 (made up of figures 11A and 11B) is a detailed description of the *Hind*III fragment insert in Homology Vector 556-41.5. The diagram shows the orientation of DNA fragments assembled in the cassette. The origin of each fragment is described in the Materials and Methods section. The sequences located at the junctions between each fragment

and at the ends of the marker gene are shown, including junction A (SEQ ID NO: 37), junction B (SEQ ID NO: 38), and junction C (SEQ ID NO: 39). The restriction sites used to generate each fragment are indicated at the appropriate junction. The location of the MDV gD and a portion of the gI gene is also given. Numbers in parenthesis () refer to amino acids, and restriction sites in brackets [] indicate the remnants of sites which were destroyed during construction.

Figure 12 (made up of figures 12A, 12B, and 12C) is a detailed description of the *SalI* fragment insert in Homology Vector 255-18.B16. The diagram shows the orientation of DNA fragments assembled in the cassette. The origin of each fragment is described in the Materials and Methods section. The sequences located at the junctions between each fragment and at the ends of the marker gene are shown, including junction A (SEQ ID NO: 40), junction B (SEQ ID NO: 41), junction C (SEQ ID NO: 42), junction D (SEQ ID NO: 43), junction E (SEQ ID NO: 44), junction F(SEQ ID NO: 45), junction G (SEQ ID NO: 46), and junction H (SEQ ID NO: 47). The restriction sites used to generate each fragment are indicated at the appropriate junction. The location of the NDV F and *lacZ*-NDV HN hybrid gene are shown. Numbers in parenthesis () refer to amino acids, and restriction sites in brackets [] indicate the remnants of sites which were destroyed during construction.

Detailed Description Of The Invention

The present invention provides a recombinant herpesvirus of turkeys comprising a foreign gene inserted within the genomic DNA of herpesvirus of turkeys, wherein the foreign gene is inserted into the unique *StuI* site in the US2 gene coding region of the genomic DNA and is capable of being expressed in a host cell infected with herpesvirus of turkeys.

For purposes of this invention, "genomic DNA" means the entire DNA which the naturally occurring herpesvirus of turkeys contains. For purposes of this invention, "foreign gene" means a gene which is exogenous to the herpesvirus of turkeys genomic DNA.

For purposes of this invention, a "foreign gene" includes not only a DNA sequence corresponding to a polypeptide coding region, but also a "promoter" located upstream of said DNA sequence. For purposes of this invention, a "promoter" is a specific DNA sequence on the DNA molecule to which the foreign RNA polymerase attaches and at which transcription of the foreign RNA is initiated.

The invention further provides recombinant herpesviur sof turkeys wherein the foreign gene inserted into the unique *StuI* site in the US2 gene coding region of the HVT genomic DNA and is capable of being expressed in a host cell infected with herpesvirus of turkeys encodes a polypeptide.

In one embodiment of the invention, the polypeptide is *E. coli* beta-galactosidase. Preferably, this recombinant herpesvirus of turkeys is designated or S-HVT-012. The S-HVT-012 herpesvirus has been deposited on October 15, 1992

pursuant to the Budapest Treaty on the International Deposit of Microorganism for the Purposes of Patent Procedure with the Patent Culture Depository of the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852 U.S.A. under ATCC Accession No. VR 2382.

In another embodiment of the invention, the polypeptide is antigenic in the animal into which the recombinant herpesvirus of turkeys is introduced. The gene coding for such antigenic polypeptide can be derived from Mareks's disease virus, Newcastle disease virus, infectious laryngotracheitis virus, infectious bronchitis virus or infectious bursal disease virus.

The invention further provides a recombinant herpesvirus of turkeys whose genomic DNA contains a foreign gene encoding antigenic polypeptide from Marek's disease virus, wherein the foreign gene is inserted into the unique *StuI* site in the US2 gene coding region of the HVT genomic DNA and is capable of being expressed in a host cell infected with herpesvirus of turkeys encodes a polypeptide. Preferably, this antigenic polypeptide is Marek's disease virus glycoprotein gB, gA or gD.

In one embodiment of the above invention, the Marek's disease virus gene inserted into the unique *StuI* site in the US2 gene coding region of the HVT genomic DNA encodes Marek's disease virus glycoprotein gB. Preferably, this recombinant herpesvirus of turkeys is designated S-HVT-045. The S-HVT-045 herpesvirus has been deposited on October 15, 1992 pursuant to the Budapest Treaty on the International Deposit of Microorganism for the Purposes of Patent Procedure with the Patent Culture Depository of the American Type Culture Collection, 12301 Parklawn Drive, Rockville,

Maryland 20852 U.S.A. under ATCC Accession No. VR 2383.

More than one Marek's virus antigen coding gene can be inserted into the herpesvirus of turkeys genomic DNA. For example, a gene encoding Marek's disease virus glycoprotein gB and a gene encoding Marek's disease virus glycoprotein gA can both be inserted into the HVT genome. In such an instance, it is not necessary for purposes of this invention that both of these genes be inserted into the unique *StuI* site in the US2 gene coding region. All that is required for this invention is that one of them is. The other one can be inserted into another non-essential region of the HVT genome, such as Bam #16 region or EcoRI #9 region. However, a preferable embodiment of this invention is a recombinant herpesvirus of turkeys containing both Marek's disease virus glycoprotein gA gene and Marek's disease virus glycoprotein gB gene inserted into the unique *StuI* site in the US2 gene coding region. Preferably, this recombinant herpesvirus of turkeys is designated S-HVT-046 or S-HVT-047.

One embodiment of the above invention further provides a recombinant herpesvirus of turkeys containing all three of the Marek's disease virus genes encoding antigenic polypeptide: Marek's disease virus glycoprotein gA gene, glycoprotein gB gene and glycoprotein gD gene. For purposes of this invention, not all three of these genes have to be inserted into the unique *StuI* site in the US2 gene coding region. It is sufficient that one of them is. However, a preferred embodiment of the invention does have all three Marek's disease virus antigen coding genes inserted into the unique *StuI* site in the US2 gene coding region. Preferably, this recombinant herpesvirus of turkeys is S-HVT-062.

The invention further provides a recombinant herpesvirus of turkeys whose genomic DNA contains a foreign gene encoding

antigenic polypeptide from Newcastle disease virus, wherein the foreign gene is inserted into the unique *StuI* site in the US2 gene coding region of the HVT genomic DNA and is capable of being expressed in a host cell infected with herpesvirus of turkeys. Preferably, this Newcastle disease virus gene encodes Newcastle disease virus fusion protein or Newcastle disease virus hemagglutinin-neuraminidase.

The recombinant herpesvirus of turkeys of the above invention can be engineered further to also contain a gene encoding Marek's disease virus antigenic polypeptide, such as Marek's disease virus glycoprotein gA, gB or gD. For purposes of the present invention, as long as one of these antigen coding genes, whether from Marek's disease virus or from Newcastle disease viurs, is inserted into the the unique *StuI* site in the US2 gene coding region of the HVT genomic DNA, it is not necessary that the other antigenic genes are also inserted into that site. It is sufficient that the other antigenic genes from Marek's disease virus or from Newcastle disease virus are inserted in another nonessential region of the HVT genomic DNA, such as the BamHI #16 region or the EcoRI #9 region.

In one embodiment of the above invention, a recombinant herpesvirus of turkeys contains three foreign genes inserted into the unique *StuI* site in the US2 gene coding region of the HVT genomic DNA : a gene encoding Newcastle disease virus fusion protein, a gene encoding Marek's disease virus glycoprotein gB and a gene encoding Marek's disease virus glycoprotein gA. Preferably, this recombinant herpesvirus of turkeys is designated S-HVT-048.

In another embodiment of the above invention, a recombinant herpesvirus of turkeys contains a different combination of three foreign genes inserted into the unique *StuI* site in

the US2 gene coding region of the HVT genomic DNA : a gene encoding Newcastle disease hemagglutinin-neuraminidase, a gene encoding Marek's disease virus glycoprotein gB and a gene encoding Marek's disease virus glycoprotein gA. Preferably, this recombinant herpesvirus of turkeys is designated S-HVT-049.

In another embodiment of the above invention, a recombinant virus of turkeys contains the following Marek's disease virus genes and Newcastle disease virus genes, all of them inserted into the unique *StuI* site in the US2 gene coding region of the HVT genomic DNA : a gene encoding Newcastle disease virus fusion protein, a gene encoding Newcastle disease hemagglutinin-neuraminidase, a gene encoding Marek's disease virus glycoprotein gB and a gene encoding Marek's disease virus glycoprotein gA. Preferably, this recombinant herpesvirus of turkeys is designated S-HVT-050.

In yet another embodiment of the above invention, a recombinant virus of turkeys contains the following five genes from Marek's disease virus and Newcastle disease virus, all of them inserted into the unique *StuI* site in the US2 gene coding region of the HVT genomic DNA : a gene encoding Newcastle disease virus fusion protein, a gene encoding Newcastle disease hemagglutinin-neuraminidase, a gene encoding Marek's disease virus glycoprotein gB, a gene encoding Marek's disease virus glycoprotein gA and a gene encoding Marek's disease virus glycoprotein gD. Preferably, this recombinant herpesvirus of turkeys is designated S-HVT-106.

The invention further provides a recombinant herpesvirus of turkeys whose genomic DNA contains a foreign gene encoding antigenic polypeptide from infectious laryngotracheitis virus, wherein the foreign gene is inserted into the unique

StuI site in the US2 gene coding region of the HVT genomic DNA and is capable of being expressed in a host cell infected with herpesvirus of turkeys.. Preferably, this infectious laryngotracheitis virus gene encodes infectious laryngotracheitis virus glycoprotein gB or infectious laryngotracheitis virus glycoprotein gD.

In one embodiment of the above invention, a recombinant herpesvirus of turkeys contains infectious laryngotracheitis virus glycoprotein gB gene inserted into the unique *StuI* site in the US2 gene coding region of the HVT genomic DNA. Preferably, this recombinant herpesvirus of turkeys is designated S-HVT-051.

In another embodiment of the above invention, a recombinant herpesvirus of turkeys contains infectious laryngotracheitis virus glycoprotein gD gene inserted into the *StuI* site in the US2 gene coding region of the HVT genomic DNA. Preferably, this recombinant herpesvirus of turkeys is designated S-HVT-052.

The recombinant herpesvirus of turkeys of the above invention can be engineered further to contain, in addition to a gene encoding infectious laryngotracheitis virus glycoprotein gB or infectious laryngotracheitis virus glycoprotein gD or both, one or more genes encoding Marek's disease virus antigenic polypeptide, such as Marek's disease virus glycoprotein gA, gB or gD. For purposes of the present invention, as long as one of these antigen coding genes, whether it is from Marek's disease virus or from infectious laryngotracheitis virus, is inserted into the unique *StuI* site in the US2 gene coding region of the HVT genomic DNA, it is not necessary that the other antigenic genes are also inserted into that site. It is sufficient that the other antigenic genes from Marek's disease virus or

infectious laryngotracheitis virus are inserted in another nonessential region of the HVT genomic DNA, such as the BamHI #16 region or the EcoRI #9 region.

The invention further provides a recombinant herpesvirus of turkeys whose genoic DNA contains one or more foreign gene encoding antigneic polypeptide from infectious bronchitis virus, wherein the foreign gene is inserted into the unique *Stu*I site in the US2 gene coding region of the HVT genomic DNA and is capable of being expressed in a host cell infected with herpesvirus of turkeys. Preferably, this infectious bronchitis virus gene encodes infectious bronchitis virus spike protein or infectious bronchitis virus matrix protein.

The recombinant herpesvirus of turkeys of the above invention can be engineered further to contain, in addition to a gene encoding infectious bronchitis virus spike protein or infectious bronchitis virus glycoprotein matrix protein or both, one or more genes encoding Marek's disease virus antigenic polypeptide, such as Marek's disease virus glycoprotein gA, gB or gD. For purposes of the present invention, as long as one of these antigen coding genes, whether it is from Marek's disease virus or from infectious bronchitis virus, is inserted into the unique *Stu*I site in the US2 gene coding region of the HVT genomic DNA, it is not necessary that the other antigenic genes are also inserted into that site. It is sufficient that the other antigenic genes from Marek's disease virus or infectious bronchitis virus are inserted in another nonessential region of the HVT genomic DNA, such as the BamHI #16 region or the EcoRI #9 region.

In one embodiment of the above invention, a recombinant

herpesvirus of turkeys contains the following five foreign genes inserted into the unique *StuI* site in the US2 gene coding region of the HVT genomic DNA : a gene encoding infectious bronchitis matrix protein, a gene encoding infectious bronchitis spike protein, a gene encoding Marek's disease virus glycoprotein gA, a gene encoding Marek's disease virus glycoprotein gB, and a gene encoding Marek's disease virus glycoprotein gD. Preferably, this recombinant herpesvirus of turkeys is designated S-HVT-066.

The invention further provides a recombinant herpesvirus of turkeys whose genoic DNA contains one or more foreign gene encoding antigneic polypeptide from infectious bursal disease virus, wherein the foreign gene is inserted into the unique *StuI* site in the US2 gene coding region of the HVT genomic DNA and is capable of being expressed in a host cell infected with herpesvirus of turkeys. Preferably, this infectious bursal disease virus gene encodes infectious bursal disease virus VP2 protein. Preferably, this recombinant herpesvirus of turkeys is designated S-HVT-096.

The recombinant herpesvirus of turkeys of the above invention can be engineered further to contain, in addition to a gene encoding infectious bursal disease virus VP2 protein, one or more genes encoding Marek's disease virus antigenic polypeptide, such as Marek's disease virus glycoprotein gA, gB or gD. For purposes of the present invention, as long as one of these antigen coding genes, whether it is from Marek's disease virus or from infectious bursal disease virus, is inserted into the unique *StuI* site in the US2 gene coding region of the HVT genomic DNA, it is not necessary that the other antigenic genes are also inserted into that site. It is sufficient that the other antigenic genes from Marek's disease virus or infectious

bursal disease virus are inserted in another nonessential region of the HVT genomic DNA, such as the BamHI #16 region or the EcoRI #9 region.

The inserted foreign gene includes a promoter sequence which controls its expression. Preferably, the promoter is a herpesvirus promoter. Preferably, the promoter is selected from PRV gX, HSV-1 alpha 4, HCMV immediate early, MDV gB, MDV gD, ILT gB, and ILT gD. For purposes of this invention, the promoters were generated by methods well known to those skilled in the art.

The invention provides for a homology vector for producing a recombinant herpesvirus of turkeys by inserting foreign DNA into the genomic DNA of a herpesvirus of turkeys. The homology vector comprises a double-stranded DNA molecule consisting essentially of a double-stranded foreign DNA not usually present within the herpesvirus of turkeys genomic DNA, with at one end of the foreign DNA, double-stranded herpesvirus of turkeys DNA homologous to genomic DNA located at one side of the unique StuI site of the US2 gene coding region of the herpesvirus of turkeys, and at the other end of the foreign DNA, double-stranded herpesvirus of turkeys DNA homologous to genomic DNA located at the other side of the same site on the genomic DNA.

In one embodiment of the invention, the foreign DNA encodes a polypeptide. In one embodiment of the invention, the polypeptide is antigenic in the animal into which the recombinant herpesvirus of turkeys is introduced. Preferably, the antigenic polypeptide is from infectious bursal disease virus, Marek's disease virus, Newcastle disease virus, infectious laryngotracheitis virus, or infectious bronchitis virus. Preferably, the antigenic

polypeptide is selected from a group consisting of infectious bursal disease virus VP2 protein, infectious bursal disease virus VP3 protein, infectious bursal disease virus VP4 protein, Marek's disease virus glycoprotein gB, Marek's disease virus glycoprotein gA, Marek's disease virus glycoprotein gD, Newcastle disease virus fusion (F) protein, Newcastle disease virus hemagglutinin-neuraminidase (HN) protein, infectious laryngotracheitis virus glycoprotein gB, infectious laryngotracheitis virus glycoprotein gD, infectious bronchitis virus spike protein, or infectious bronchitis virus matrix protein.

In one embodiment of the invention, the polypeptide is a detectable marker. Preferably, the polypeptide which is a detectable marker is *E. coli* beta-galactosidase.

For purposes of this invention, a "homology vector" is a plasmid constructed to insert foreign DNA in a specific site on the genome of a herpesvirus of turkeys.

In one embodiment of the invention, the double-stranded herpesvirus of turkeys DNA is homologous to DNA sequences present within the US2 gene coding region of the herpesvirus of turkeys genome. Preferably, this homology vector is designated 435-47.1.

The invention further provides a vaccine which comprises an effective immunizing amount of a recombinant herpesvirus of turkeys of the present invention and a suitable carrier.

Suitable carriers for the herpesvirus of turkeys are well known in the art and include proteins, sugars, etc. One example of such a suitable carrier is a physiologically balanced culture medium containing one or more stabilizing agents such as stabilized, hydrolyzed proteins, lactose,

etc.

For purpose of this invention, an "effective immunizing amount" of recombinant herpesvirus of the present invention is within the range of 10^2 to 10^9 PFU/dose.

The present invention also provides a method of immunizing a fowl. For purposes of this invention, this includes immunizing a fowl against infectious bursal disease virus, Marek's disease virus, Newcastle disease virus, infectious laryngotracheitis virus, or infectious bronchitis virus. The method comprises administering to the fowl an effective immunizing dose of the vaccine of the present invention. The vaccine may be administered by any of the methods well known to those skilled in the art, for example, by intramuscular, subcutaneous, intraperitoneal or intravenous injection. Alternatively, the vaccine may be administered intranasally or orally.

The present invention also provides a host cell infected with a recombinant herpesvirus of turkeys. Preferably, the host cell is an avian cell.

For purposes of this invention, a "host cell" is a cell used to propagate a vector and its insert. Infecting the cell was accomplished by methods well known to those skilled in the art, for example, as set forth in DNA TRANSFECTION FOR GENERATING RECOMBINANT HERPESVIRUS in Materials and Methods.

A recombinant herpesvirus of turkeys of the present invention provide a way for distinguishing an animal vaccinated with the vaccine of the present invention from an animal infected with a naturally-occurring, wild-type infectious bursal disease virus, Marek's disease virus, Newcastle disease virus, infectious laryngotracheitis virus,

or infectious bronchitis virus. This is possible because recombinant herpesvirus of turkeys contain foreign DNA which encodes a limited number of antigens from the above mentioned viruses that are needed to confer protective immunity to the corresponding pathogens. Consequently, host animals vaccinated with the recombinant herpesvirus of turkeys can be distinguished from those which have been infected with wild-type infectious bursal disease virus, Marek's disease virus, Newcastle disease virus, infectious laryngotracheitis virus, or infectious bronchitis virus by the absence of antigens that are normally present in the wild type viruses.

Methods for constructing, selecting and purifying recombinant herpesvirus of turkeys are detailed below in Materials and Methods.

Materials and Methods

PREPARATION OF HERPESVIRUS OF TURKEYS STOCK SAMPLES. Herpesvirus of turkeys stock samples were prepared by infecting tissue culture cells at a multiplicity of infection of 0.01 PFU/cell in Dulbecco's Modified Eagle Medium (DMEM) containing 2 Mm glutamine, 100 units/ml penicillin, 100 units/ml streptomycin (these components are obtained from Irvine Scientific or an equivalent supplier, and hereafter are referred to as complete DMEM medium) plus 2% fetal bovine serum. After cytopathic effect was complete, the medium and cells were harvested and the cells were pelleted at 3000 rpm for 5 minutes in a clinical centrifuge. Infected cells were resuspended in complete medium containing 20% fetal bovine serum, 10% DMSO and stored frozen at -70°C.

PREPARATION OF HERPESVIRUS OF TURKEY DNA. All manipulations of herpesvirus of turkey (HVT) were made using strain FC-126 (ATCC #584-C). For the preparation of HVT viral DNA from the cytoplasm of infected cells, primary chicken embryo fibroblasts were infected at a MOI sufficient to cause extensive cytopathic effect before the cells overgrew. All incubations were carried out at 39°C in a humidified incubator with 5% CO_2 in air. Best DNA yields were obtained by harvesting monolayers which were maximally infected, but showing incomplete cell lysis (typically 5-7 days). Infected cells were harvested by scraping the cells into the medium using a cell scraper (Costar brand). The cell suspension was centrifuged at 3000 rpm for 10 minutes at 5°C in a GS-3 rotor (Sorvall Instruments). The resultant pellet was resuspended in cold PBS (20 ml/Roller Bottle) and subjected to another centrifugation for 10 minutes at 3000 rpm in the cold. After decanting the PBS, the cellular pellet was resuspended in 4 ml/roller bottle of RSB buffer

(10 mM Tris pH 7.5, 1 mM EDTA, and 1.5 mM $MgCl_2$). NP40 (Nonidet P-40™;Sigma) was added to the sample to a final concentration of 0.5% and allowed to incubate on ice for 15 minutes with occasional mixing. The sample was centrifuged for 10 minutes at 3000 rpm in the cold to pellet the nuclei and remove cellular debris. The supernatant fluid was carefully transferred to a 15 ml Corex centrifuge tube. Both EDTA (0.5M pH 8.0) and SDS (sodium dodecyl sulfate; stock 20%) were added to the sample to final concentrations of 0.5M and 1%, respectively. One hundred µl of proteinase-K (10 mg/ml; Boehringer Mannheim) was added per 4 ml of sample, mixed, and incubated at 45°C for 1-2 hours. After this period, an equal volume of water-saturated phenol was added to the sample and gently mixed by hand. The sample was spun in a clinical centrifuge for 5 minutes at 3000 rpm to separate the phases. NaAc was added to the aqueous phase to a final concentration of 0.3M (stock solution 3M pH 5.2), and the nucleic acid precipitated at -70°C for 30 minutes after the addition of 2.5 volumes of cold absolute ethanol. DNA in the sample was pelleted by spinning for 20 minutes to 8000 rpm in an HB-4 rotor at 5°C. The supernatant was carefully removed and the DNA pellet washed once with 25 ml of 80% ethanol. The DNA pellet was dried briefly by vacuum (2-3 minutes), and resuspended in 50 µl/roller bottle of infected cells of TE buffer (10 mM Tris pH 7.5, 1 mM EDTA). Typically, yields of viral DNA ranged between 5-10 µg/roller bottle of infected cells. All viral DNA was stored at approximately 10°C.

POLYMERASE FILL-IN REACTION. DNA was resuspended in buffer containing 50 mM Tris pH 7.4, 50 mM KCl, 5 mM $MgCl_2$, and 400 micromolar each of the four deoxynucleotides. Ten units of Klenow DNA polymerase (BRL) were added and the reaction was allowed to proceed for 15 minutes at room temperature. The DNA was then phenol extracted and ethanol precipitated as above.

DNA SEQUENCING. Sequencing was performed using the USB Sequenase Kit and ^{35}S-dATP (NEN). Reactions using both the dGTP mixes and the dITP mixes were performed to clarify areas of compression. Alternatively, compressed areas were resolved on formamide gels. Templates were double-stranded plasmid subclones or single stranded M13 subclones, and primers were either made to the vector just outside the insert to be sequenced, or to previously obtained sequence. Sequence obtained was assembled and compared using Dnastar software. Manipulation and comparison of sequences obtained was performed with Superclone and Supersee programs from Coral Software.

MOLECULAR BIOLOGICAL TECHNIQUES. Techniques for the manipulation of bacteria and DNA, including such procedures as digestion with restriction endonucleases, gel electrophoresis, extraction of DNA from gels, ligation, phosphorylation with kinase, treatment with phosphatase, growth of bacterial cultures, transformation of bacteria with DNA, and other molecular biological methods are described by Maniatis *et al* (1982) and Sambrook *et al* (1989). The polymerase chain reaction (PCR) was used to introduce restriction sites convenient for the manipulation of various DNAs. The procedures used are described by Innis *et al* (1990). In general amplified fragments were less than 500 base pairs in size and critical regions of amplified fragments were confirmed by DNA sequencing. Except as noted, these techniques were used with minor variation.

SOUTHERN BLOTTING OF DNA. The general procedure for Southern blotting was taken from Maniatis *et al*. (1982). DNA was blotted to nitrocellulose filters (S&S BA85) in 20X SSC (1X ssc = 0.15M NaCl, 0.015M sodium citrate, pH 7.0), and prehybridized in hybridization solution consisting of 30% formamide, 1X Denhardt's solution (0.02%

polyvinylpyrrolidone (PVP), 0.02% bovine serum albumin (BSA), 0.02% Ficoll), 6X SSC, 50 mM NaH_2PO_4, pH 6.8, 200 µg/ml salmon sperm DNA for 4-24 hours at 55°C. Labeled probe DNA was added that had been labeled by nick translation using a kit from Bethesda Research Laboratories (BRL) and one ^{32}P-labeled nucleotide. The probe DNA was separated from the unincorporated nucleotides by NACS column (BRL) or on a Sephadex G50 column (Pharmacia). After overnight hybridization at 55°C, the filter was washed once with 2X SSC at room temperature followed by two washes with 0.1X SSC, 0.1% sodium dodecyl sulfate (SDS) for 30 minutes at 55°C. The filter was dried and autoradiographed.

cDNA CLONING PROCEDURE. cDNA cloning refers to the methods used to convert RNA molecules into DNA molecules following state of the art procedures. Applicants' methods are described in (Gubler and>

^Z^AdVT virus is dependent upon homologous recombination between HVT viral DNA and the plasmid homology vector containing the desired foreign DNA flanked by the appropriate herpesvirus cloned sequences. Transfections were carried out in 6 cm plates (Corning plastic) of 50% confluent primary chick embryo fibroblast (CEF) cells. The cells were plated out the day before in CEF growth media (1X F10/199, 5% fetal calf serum, 2% glutamine, 1% non-essential amino acids, and 2% penicillin/streptomycin) containing 4 µg/ml polybrene (stock 4 mg/ml in 1X HBSS). For cotransfections into CEF cells, 5 µg of the plasmid homology vector was mixed with 5 µg of intact HVT DNA, and suspended in 1 ml of CEF media containing 30 µg/ml polybrene (stock 4 mg/ml in 1X HBSS). The DNA-polybrene suspension (1 ml) was then added to a 6 cm plate of CEF cells from which the media had been aspirated, and incubated at 39°C for 30 minutes. The plates were rocked periodically during this time to redistribute the inoculum. After this period, 4 ml

of CEF growth media was added directly to wash plate, and incubated an additional 2.5 hours at 39°C. At this time, the media was removed from each plate, and the cells shocked with 2 ml of 30% DMSO (Dimethyl Sulfoxide, J.T. Baker Chemical Co.) in 1X HBSS for 4 minutes at room temperature. The 30% DMSO was carefully removed and the monolayers washed once with 1X HBSS at room temperature. The cells were then incubated at 39°C after the addition of 5 mls of CEF growth media. The next day, the media was changed to remove any last traces of DMSO and to stimulate cell growth. Cytopathic effect from the virus becomes apparent within 6 days. Generation of a high titer stock (80%-90% CPE) can usually be made within 1 week from this date. HVT stock samples were prepared by resuspending the infected cells in CEF growth media containing 20% fetal calf serum, 10% DMSO and stored at -70°C.

PROCEDURE POR GENERATING RECOMBINANT HERPESVIRUS FROM SUBGENOMIC DNA FRAGMENTS. The ability to generate herpesviruses by cotransfection of cloned overlapping subgenmoic fragments has been demonstrated for pseudorabies virus (Zijl et al., 1988). If deletions and/or insertions are engineered directly into the subgenomic fragments prior to the cotransfection, this procedure results in a high frequency of viruses containing the genomic alteration, greatly reducing the amount of screening required to purify the recombinant virus. We have used this procedure to construct recombinant HVT.

A library of subclones containing overlapping HVT subgenomic fragments was generated as follows. HVT DNA was obtained from the American Type Culture Collection (FC-126("Calnek")). It was sheared and then size selected on a glycerol gradient as described by van Zijl *et al.*,(1988) with 40-50 kb fragments chosen as the insert population.

The pooled fractions were diluted twofold with TE, one-tenth volume of 3M NaAc and 2.5 volumes of ethanol were added, and the DNA was precipitated at 30K rpm in a Beckman SW41 rotor for 1 hr. The sheared fragments were given blunt ends by initial treatment with T4 DNA polymerase, using low DNTP concentrations to promote 3' overhang removal, followed by treatment with Klenow polymerase to fill in recessed 3' ends. These insert fragments were then ligated to a pWE15 (Strategene) cosmid vector, which had been digested with *BamHI*, treated with calf intestinal phosphatase, and made blunt by treatment with Klenow polymerase. The ligated mixture was then packaged using Gigapack XL packaging extracts (Stratagene). Ligation and packaging was as recommended by the manufacturer.

Published restriction maps for the enzymes *BamHI*, *HindIII*, and *XhoI* permitted the use of subcloned fragments as specific probes to screen the cosmid library for subclones spanning the genome. Probes were generated from subcloned restriction fragments. The fragments were then labeled using a non-radioactive system (Genius, Boehringer Mannheim). Screening was facilitated by picking colonies to media followed by growth overnight. Sets of five filters and a master plate were stamped from microtiter dish and again grown overnight. Glycerol was added to the wells to 15% and the plates were frozen at -20°C to provide stock cultures of each colony. Filters were BioRad Colony Lift Membranes and were treated and hybridized per manufacturer's instructions, and washed in 0.1X SSC, 0.1% SDS, 65°C. Clones which hybridized with the non-radioactive probe were detected according to the Genius kit directions.

Colonies were selected for further analysis on the basis of their hybridization to two or more of the specific probes. These were then digested with *BamHI*, and compared to

published maps of HVT (Buckmaster *et al.*, 1988). The three cosmids (407-32.2C3,407-32.IG7, and 407-32.5G6) were obtained in this manner. A detailed description of each clone is given below. It was found that chloramphenicol amplification (Maniatis *et al.*,1982) was necessary to achieve reasonable yields of DNA from these clones. In addition, one cosmid clone (407-32.5G6) was unstable and had to be grown from the original frozen stock in order to obtain satisfactory DNA preparations.

The pWE15 vector allows the inserts to be excised with *Not*I. However, four *Not*I sites are present in the HVT genome, so that inserts spanning these sites cannot be excised with *Not*I. Two of the *Not*I sites are present in the *Bam*HI #2 fragment of HVT, this fragment was cloned directly in pSP64. The other two sites are present in the unique short region within the *Bam*HI #1 fragment. This fragment was cloned directly in the pWE15 vector. The three sheared cosmids and the two *Bam*HI fragments cover all but a small portion of the ends of the HVT genome. Because these regions are repeated in the internal portions of the genome, all of the genetic information is available.

A *Stu*I site within the HVT US2 gene was established as a useful site for foreign DNA insertion utilizing the HOMOLOGOUS RECOMBINATION PROCEDURE FOR GENERATING RECOMBINANT HERPESVIRUSES (see Example 6). The HVT US2 gene is located within the *Bam*HI #1 fragment which contains five *Stu*I sites. To facilitate the use of this site for insertion of foreign DNA by the *Stu*I site within the US2 gene was converted to a unique *Hin*dIII site. This was accomplished by partially digesting the *Bam*HI #1 subclone with *Stu*I, and then inserting a 10 kb fragment conferring kanomycin resistance (Neo^R) into the site using *Hin*dIII linkers. The

kanomycin resistance gene allowed positive selection of recombinant clones. The Neo^R fragment was removed by digestion with *Hin*dIII followed by religation generating clone 430-84.215.

DNA was prepared for reconstruction experiments by restriction digestion with enzymes which cut the subclones outside or flanking the HVT insertions. In some instances, one cosmid in a reconstruction was used undigested. Digested DNAs were extracted once with phenol and precipitated with ethanol. DNA was resuspended at a concentration of 0.5 to 1 ug/ml. Viral reconstruction experiments were performed using Lipofectin (BRL) to mediate transfection. Two to three micrograms each of subclone were added to 0.5 ml of MEM media (Earle's salts) supplemented with 1% non-essential amino acids and 2% penicillin/Streptomysin (MEM+). Controls consisted of MEM+ with no DNA, with several ug of HVT DNA, or with 4 out of 5 of the subclones. Separately, 30 µl of the Lipofectin were added to another 0.5 ml. of MEM+. These two mixtures were then combined and incubated at RT for 15 minutes.

Chick embryo fibroblast (CEF) cells were prepared for transfection in the following manner. CEFs (Spafas) were grown in 6 well dishes at 39°C in F10/M199 (1:1) media containing 1% non-essential amino acids, 2% penicillin/streptomycin, and 5% fetal calf serum (CEF+). Cells were transfected at a confluence of 90 - 95%. For transfection, wells were aspirated and rinsed 3 times with MEM+, and then incubated 4 hours at 39°C with the 1 ml lipofectin/DNA mixture above. One ml more of CEF+ was then added to the wells, and cells were incubated overnight and fed with CEF+. Plates were then examined daily for the appearance of plaques.

Lipofectin with control HVT DNA resulted in the appearance of plaques within 5 days. When only four of the five subclones were used, no plaques were obtained. When the five overlapping genomic fragments of HVT were used to reconstruct the virus, plaques appeared anywhere from 5 to 19 days after the initial lipofection. In the case of plaques appearing late, plaques were not initially seen on the infected monolayer, and it was only after passaging the monolayer and replating on a larger surface that plaques appeared. After passaging, plaques generally appeared within 3 days. Recombinant viruses were plaque purified approximately three and then analyzed for insertion of foreign DNAs.

BLUOGAL SCREEN FOR RECOMBINANT HERPESVIRUS. When the foreign gene encoded the enzyme β-galactosidase, the plaques that contained the gene were visualized more easily. The chemical Bluogal™ (Bethesda Research Labs) was incorporated at the level of 200-300 μg/ml into the agarose overlay during the plaque assay, and the plaques that expressed active β-galactosidase turned blue. The blue plaques were then picked and purified by further blue plaque isolations. Other foreign genes were inserted by homologous recombination such that they replaced the β-galactosidase gene; in this instance non-blue plaques were picked for purification of the recombinant virus.

SCREEN FOR FOREIGN GENE EXPRESSION IN RECOMBINANT HVT USING BLACK PLAQUE ASSAYS. To analyze expression of foreign antigens expressed by recombinant HVT viruses, monolayers of CEF cells are infected with recombinant HVT, overlaid with nutrient agarose media and incubated for 4-5 days at 39°C. Once plaques have developed, the agarose overlay is removed from the dish, the monolayer rinsed 1x with PBS, fixed with 100% methanol for 10 minutes at room temperature and the

cells air dried. After re-hydrating the plate with PBS, the primary antibody is diluted to the appropriate dilution with PBS and incubated with the cell monolayer for 2 hours to overnight at room temperature. Unbound antibody is then removed from the cells by washing three times with PBS at room temperature. An alkaline phosphatase conjugated secondary antibody is diluted with PBS and incubated with the cells for 2 hours at room temperature. Unbound secondary antibody is then removed by washing the cells three times with PBS at room temperature. Next, the monolayer is rinsed in color development buffer (100mM Tris pH 9.5/ 100mM NaCl/ 5mM MgCl2), and then incubated 10 minutes to overnight at room temperature with freshly prepared substrate solution (0.3 mg/ml Nitro Blue tetrazolium + 0.15 mg/ml 5-Bromo-4-Chloro-3-Indolyl Phosphatase in color development buffer.) Finally, the reaction is stopped by replacing the substrate solution with TE (10mM Tris, pH7.5/ 1 mM EDTA). Plaques expressing the correct antigen will stain black.

PLAQUE HYBRIDIZATION PROCEDURE FOR ASSESSING THE PURITY OF RECOMBINANT HVT STOCKS: When no suitable immunological reagent exists to detect the presence of a particular antigen in a recombinant HVT virus, plaque hybridization can be used to assess the purity of a stock. Initially, CEF cell monolayers are infected with various dilutions of the viral stocks to give ~50-100 plaques/10 cm.dish, overlaid with nutrient agarose media and incubated for 4-5 days at 39°C. Once plaque development occurs, the position of each plaque is marked on bottom of the dish. The agarose overlay is then removed, the plate washed with PBS, and the remaining CEF monolayer is transferred to a NC membrane or BioRad nylon membrane pre-wetted with PBS (making note of the membrane position relative to the dish). Cells contained on the NC membranes are then lysed by placing the

membranes in 1.5 mls of 1.5M NaCl and 0.5M NaOH for five minutes. The membranes are neutralized by placing them in 1.5 mls of 3M Sodium acetate (pH 5.2) for five minutes. DNA from the lysed cells is then bound to the NC membranes by baking at 80°C for one hour. After this period the membranes are prehybridized in a solution containing 6X SSC, 3% skim milk, 0.5% SDS, (±) salmon sperm DNA (50 µg/ml) for one hour at 65°C. Radio-labeled probe DNA (alpha 32P-dCTP) is then added and the membranes incubated at 65°C overnight (~12 hours). After hybridization the NC membranes are washed two times (30 minutes each) with 2X SSC at 65°C, followed by two additional washes at 65°C with 0.5X SSC. The NC membranes are then dried and exposed to X-ray film (Kodak X-OMAT,AR) at -70°C for 12 hours. Positive signals are then aligned with the position of the plaques on the dish and purity of the stock is recorded as the percentage of positive plaques over the total.

CONSTRUCTION OF HOMOLOGY VECTOR FOR INSERTION OF THE BETA-GALACTOSIDASE GENE INTO HVT US2 GENE. The beta-galactosidase (*lacZ*) gene was inserted into the HVT *Eco*RI # 7 fragment at the unique *Stu*I site. The marker gene is oriented in the same direction as the US2 gene. A detailed description of the marker gene is given in FIGURE 7. It is constructed utilizing standard recombinant DNA techniques (Maniatis *et al*, 1982 and Sambrook *et al*, 1989), by joining restriction fragments from the following sources with the synthetic DNA sequences indicated in FIGURE 7. Fragment 1 is an approximately 413 base pair *Sal*I to *Bam*HI restriction sub-fragment of the PRV *Bam*HI restriction fragment 10 (Lomniczi et al., 1984). Fragment 2 is an approximately 3010 base pair *Bam*HI to *Pvu*II restriction fragment of plasmid pJF751 (Ferrari *et al*., 1985). Fragment 3 is an approximately 754 base pair *Nde*I to *Sal*I restriction sub-fragment of the PRV *Bam*HI restriction fragment #7 (Lomniczi

et al., 1984).

SUBGENOMIC CLONE 172-07.BA2. Plasmid 172-07.BA2 was constructed for the purpose of generating recombinant HVT. It contains an approximately 25,000 base pair region of genomic HVT DNA. It may be used in conjunction with other subgenomic clones according to the PROCEDURE FOR GENERATING RECOMBINANT HERPESVIRUS FROM OVERLAPPING SUBGENOMIC FRAGMENTS for the construction of recombinant HVT. This plasmid may be constructed utilizing standard recombinant DNA techniques (Maniatis *et al*, 1982 and Sambrook *et al*, 1989), by joining two restriction fragments from the following sources. The first fragment is an approximately 2999 base pair *BamHI* to *BamHI* restriction fragment of pSP64 (Promega). The second fragment is the approximately 25,000 base pair *BamHI* #2 fragment of HVT (Buckmaster *et al.*, 1988).

HOMOLOGY VECTOR 172-29.31. The plasmid 172-29.31 was constructed for the purpose of inserting foreign DNA into HVT. It contains a unique *XhoI* restriction enzyme site into which foreign DNA may be inserted. When a plasmid containing a foreign DNA insert at the *XhoI* site is used according to the DNA COTRANSFECTION FOR GENERATING RECOMBINANT HERPESVIRUSES or the PROCEDURE FOR GENERATING RECOMBINANT HERPESVIRUS FROM OVERLAPPING SUBGENOMIC FRAGMENTS a virus containing the foreign DNA will result. This plasmid may be constructed utilizing standard recombinant DNA techniques (Maniatis *et al*, 1982 and Sambrook *et al*, 1989), by joining two restriction fragments from the following sources. The first fragment is an approximately 2999 base pair *BamHI* to *BamHI* restriction fragment of pSP64 (Promega). The second fragment is the approximately 3300 base pair *BamHI* #16 fragment of HVT (Buckmaster *et al.*, 1988). The complete sequence of the

BamHI #16 fragment is given in SEQ ID NO:3. Note that the fragment was cloned such that the UL43 ORF is in the opposite transcriptional orientation to the pSP64 β-lacatamase gene.

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HOMOLOGY VECTOR 172-63.1. The plasmid 172-63.1 was constructed for the purpose of inserting foreign DNA into HVT. It contains a unique *Xho*I restriction enzyme site into which foreign DNA may be inserted. When a plasmid containing a foreign DNA insert at the *Xho*I site is used according to the DNA COTRANSFECTION FOR GENERATING RECOMBINANT HERPESVIRUSES or the PROCEDURE FOR GENERATING RECOMBINANT HERPESVIRUS FROM OVERLAPPING SUBGENOMIC FRAGMENTS a virus containing the foreign DNA will result. This plasmid may be constructed utilizing standard recombinant DNA techniques (Maniatis *et al*, 1982 and Sambrook *et al*, 1989), by joining two restriction fragments from the following sources. The first fragment is an approximately 2999 base pair *Eco*RI to *Eco*RI restriction fragment of pSP64 (Promega). The second fragment is the approximately 5500 base pair *Eco*RI #9 fragment of HVT. Note that the *Eco*RI fragment was cloned such that the unique *Xho*I site is closest to the unique *Hind*III site in the pSP64 vector.

HOMOLOGY VECTORS 255-18.B16. The plasmid 255-18.B16 was constructed for the purpose of inserting the NDV HN and F genes into HVT. The NDV HN and F genes were inserted as a *Sal*I fragment into the homology vector 172-29.31 at the *Xho*I site. The NDV HN and F genes were inserted in the same transcriptional orientation the UL43 ORF in the parental homology vector. A detailed description of the *Sal*I fragment is shown in figure 12. The inserted *Sal*I fragment may be constructed utilizing standard recombinant DNA techniques (Maniatis *et al*, 1982 and Sambrook *et al*, 1989), by joining restriction fragments from the following sources with the synthetic DNA sequences indicated in figure 12. Fragment 1 is an approximately 416 base pair *Sal*I to *Bam*HI restriction sub-fragment of the PRV *Bam*HI restriction fragment 10 (Lomniczi *et al*., 1984). Fragment 2 is an approximately

3009 base pair *Bam*HI to *Pvu*II fragment of the plasmid pJF751 (Ferrari *et al.*, 1985). Fragment 3 is an approximately 1200 base pair *Ava*II to *Eco*RI restriction fragment of full length NDV HN cDNA. Fragment 4 is an approximately 179 base pair *Eco*RI to *Pvu*II restriction fragment of the plasmid pSP64 (Promega). Fragment 5 is an approximately 357 base pair *Sma*I to *Bam*HI restriction sub-fragment of the HSV-1 *Bam*HI restriction fragment N. Fragment 6 is an approximately 1812 base pair *Bam*HI to *Pst*I restriction fragment of the full length NDV F cDNA. Fragment 7 is an approximately 235 base pair *Pst*I to *Sca*I restriction fragment of the plasmid pBR322.

SUBGEMOMIC CLONE 378-50.BA1. Cosmid 378-50.BA1 was constructed for the purpose of generating recombinant HVT. It contains an approximately 29,500 base pair region of genomic HVT DNA. It may be used in conjunction with other subgenomic clones according to the PROCEDURE FOR GENERATING RECOMBINANT HERPESVIRUS FROM OVERLAPPING SUBGENOMIC FRAGMENTS for the construction of recombinant HVT. This cosmid may be constructed by joining two restriction fragments from the following sources. The first fragment is an approximately 8164 base pair *Bam*HI to *Bam*HI restriction fragment of pWE15 (Stratagene). The second fragment is the approximately 29,500 base pair *Bam*HI #1 fragment of HVT (Buckmaster *et al.*, 1988).

SUBGEMOMIC CLONE 407-32.1C1. Cosmid 407-32.1C1 was constructed for the purpose of generating recombinant HVT. It contains an approximately 38,850 base pair region of genomic HVT DNA (see figure 8). This region includes *Bam*HI fragments 11, 7, 8, 21, 6, 18, approximately 1250 base pairs of fragment 13, and approximately 6,700 base pairs of fragment 1. It may be used in conjunction with other subgenomic clones according to the PROCEDURE FOR GENERATING

RECOMBINANT HERPESVIRUS FROM OVERLAPPING SUBGENOMIC FRAGMENTS for the construction of recombinant HVT. This cosmid maybe constructed as described above in the PROCEDURE FOR GENERATING RECOMBINANT HERPESVIRUS FROM OVERLAPPING SUBGENOMIC FRAGMENTS. It was isolated from the sheared DNA library by screening with the probes P1 and P4 (described in figure 8). A bacterial strain containing this cosmid has been deposited on March 3, 1993 pursuant to the Budapest Treaty on the International Deposit of Microorganisms for the Purposes of Patent Procedure with the Patent Culture Depository of the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852 U.S.A. under ATCC Accession No. 75428.

SUBGENOMIC CLONE 407-32.2C3. Cosmid 407-32.2C3 was constructed for the purpose of generating recombinant HVT. It contains an approximately 40,170 base pair region of genomic HVT DNA (see figure 8). This region includes *Bam*HI fragments 10, 14, 19, 17, 5, and approximately 2,100 base pairs of fragment 2. It may be used in conjunction with other subgenomic clones according to the PROCEDURE FOR GENERATING RECOMBINANT HERPESVIRUS FROM OVERLAPPING SUBGENOMIC FRAGMENTS for the construction of recombinant HVT. This cosmid may be constructed as described above in the PROCEDURE FOR GENERATING RECOMBINANT HERPESVIRUS FROM OVERLAPPING SUBGENOMIC FRAGMENTS. It was isolated from the sheared DNA library by screening with the probes P1 and P2 (described in figure 8). A bacterial strain containing this cosmid has been deposited pursuant to the Budapest Treaty on the International Deposit of Microorganisms for the Purposes of Patent Procedure with the Patent Culture Depository of the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852 U.S.A. under ATCC Accession No. 75430.

SUBGEMOMIC CLONE 407-32.5G6. Cosmid 407-32.5G6 was constructed for the purpose of generating recombinant HVT. It contains an approximately 40,000 base pair region of genomic HVT DNA (see figure 8). This region includes *Bam*HI fragments 9, 3, 20, 12, 16, 13, approximately 1,650 base pairs of fragment 2, and approximately 4,000 base pairs of fragment 11. It may be used in conjunction with other subgenomic clones according to the PROCEDURE FOR GENERATING RECOMBINANT HERPESVIRUS FROM OVERLAPPING SUBGENOMIC FRAGMENTS for the construction of recombinant HVT. This cosmid may be constructed as described above in the PROCEDURE FOR GENERATING RECOMBINANT HERPESVIRUS FROM OVERLAPPING SUBGENOMIC FRAGMENTS. It was isolated from the sheared DNA library by screening with the probes P2 and P3 (described in figure 8). A bacterial strain containing this cosmid has been deposited on March 3, 1993 pursuant to the Budapest Treaty on the International Deposit of Microorganisms for the Purposes of Patent Procedure with the Patent Culture Depository of the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852 U.S.A. under ATCC Accession No. 75427.

HOMOLOGY VECTOR 435-47.1. The plasmid 435-47.1 was constructed for the purpose of inserting foreign DNA into HVT. It contains a unique *Hind*III restriction enzyme site into which foreign DNA may be inserted. When a plasmid containing a foreign DNA insert at the *Hind*III site is used according to the DNA COTRANSFECTION FOR GENERATING RECOMBINANT HERPESVIRUSES or the PROCEDURE FOR GENERATING RECOMBINANT HERPESVIRUS FROM OVERLAPPING SUBGENOMIC FRAGMENTS a virus containing the foreign DNA will result. This plasmid may be constructed utilizing standard recombinant DNA techniques (Maniatis *et al*, 1982 and Sambrook *et al*, 1989), by joining two restriction fragments from the following sources. The first fragment is an

approximately 2999 base pair *Eco*RI to *Eco*RI restriction fragment of pSP64 (Promega). The second fragment is the approximately 7300 base pair *Eco*RI #7 fragment of HVT. Note that the *Hind*III site of the pSP64 vector was removed by digesting the subclone with *Hind*III followed by a Klenow fill in reaction and religation. A synthetic HindIII linker (CAAGCTTG) was then inserted into the unique *Stu*I site of the *Eco*RI #7 fragment.

SUBGEMOMIC CLONE 437-26.24. Plasmid 437-26.24 was constructed for the purpose of generating recombinant HVT. It contains an approximately 13,600 base pair region of genomic HVT DNA. It may be used in conjunction with other subgenomic clones according to the PROCEDURE FOR GENERATING RECOMBINANT HERPESVIRUS FROM OVERLAPPING SUBGENOMIC FRAGMENTS for the construction of recombinant HVT. This plasmid may be constructed utilizing standard recombinant DNA techniques (Maniatis *et al*, 1982 and Sambrook *et al*, 1989), by joining two restriction fragments from the following sources. The first fragment is an approximately 2970 base pair *Hind*III to *Bam*HI restriction fragment of pSP64 (Promega). The second fragment is the approximately 13,600 base pair *Bam*HI to *Stu*I sub-fragment of the *Bam*HI #2 fragment of HVT (Buckmaster *et al*., 1988). Note that the *Bam*HI #2 fragment contains five *Stu*I sites, the site utilized in this subcloning was converted to a *Hind*III site as described in the PROCEDURE FOR GENERATING RECOMBINANT HERPESVIRUS FROM OVERLAPPING SUBGENOMIC FRAGMENTS.

SUBGEMOMIC CLONE 437-26.26. Plasmid 437-26.26 was constructed for the purpose of generating recombinant HVT. It contains an approximately 15,300 base pair region of genomic HVT DNA. It may be used in conjunction with other subgenomic clones according to the PROCEDURE FOR GENERATING RECOMBINANT HERPESVIRUS FROM OVERLAPPING SUBGENOMIC

FRAGMENTS for the construction of recombinant HVT. This plasmid may be constructed utilizing standard recombinant DNA techniques (Maniatis *et al*, 1982 and Sambrook *et al*, 1989), by joining two restriction fragments from the following sources. The first fragment is an approximately 2970 base pair *Hind*III to *Bam*HI restriction fragment of pSP64 (Promega). The second fragment is the approximately 15,300 base pair *Bam*HI to *Stu*I sub-fragment of the *Bam*HI #2 fragment of HVT (Buckmaster *et al*., 1988). Note that the *Bam*HI #2 fragment contains five *Stu*I sites, the site utilized in this subcloning was converted to a *Hind*III site as described in the PROCEDURE FOR GENERATING RECOMBINANT HERPESVIRUS FROM OVERLAPPING SUBGENOMIC FRAGMENTS.

HOMOLOGY VECTORS 456-18.18 and 456-17.22. The plasmids 456-18.18 and 456-17.22 were constructed for the purpose of inserting the MDV gA and gB genes into HVT. The MDV genes were inserted as a cassette into the homology vector 435-47.1 at the unique *Hind*III site. The MDV genes were inserted at the blunt ended *Hind*III site as a blunt ended *Pst*I to *Eco*RI fragment (see figure 10). The *Hind*III and *Eco*RI sites were blunted by the Klenow fill in reaction. The *Pst*I site was blunted by the T4 DNA polymerase reaction. Note that the MDV cassette was inserted in both orientations. Plasmid 456-18.18 contains the MDV genes inserted in the opposite transcriptional orientation to the US2 gene in the parental homology vector. Plasmid 456-17.22 contains the MDV genes inserted in the same transcriptional orientation as the US2 gene in the parental homology vector. A detailed description of the MDV cassette is given in figure 10. It may be constructed utilizing standard recombinant DNA techniques (Maniatis *et al*, 1982 and Sambrook *et al*, 1989), by joining restriction fragments from the following sources with the synthetic DNA sequences indicated in figure 10. Fragment 1 is an approximately 2178

base pair *Pvu*II to *Eco*RV restriction sub-fragment of the MDV *Eco*RI 6.9 KB genomic restriction fragment (Ihara *et al.*, 1989). Fragment 2 is an approximately 3898 base pair *Sal*I to *Eco*RI genomic MDV fragment (Ross, et al., 1989).

HOMOLOGY VECTOR 528-03.37. The plasmid 528-03.37 was constructed for the purpose of inserting the infectious laryngotracheitis (ILT) virus gD gene into HVT. The gD gene followed by the PRV gX poly adenylation signal was inserted as a cassette into the homology vector 435-47.1 at the unique *Hin*dIII site. The cassette may be constructed utilizing standard recombinant DNA techniques (Maniatis *et al*, 1982 and Sambrook *et al*, 1989), by joining restriction fragments from the following sources. The first fragment is an approximately 2060 base pair *Eco*RI to *Bcl*I restriction sub-fragment of the ILT *Kpn*I genomic restriction fragment #8 (10.6 KB). The second fragment is an approximately 754 base pair *Nde*I to *Sal*I restriction sub-fragment of the PRV *Bam*HI restriction fragment #7 (Lomniczi *et al.*, 1984). Note that the fragments are oriented such that *Bcl*I and *Nde*I sites are contiguous.

HOMOLOGY VECTOR 528-11.43. The plasmid 528-11.43 was constructed for the purpose of inserting the infectious laryngotracheitis (ILT) virus gB gene (A.M. Grifin, 1991) into HVT. The gB gene was inserted as an *Eco*RI fragment into the homology vector 435-47.1 at the unique *Hin*dIII site. The gB gene was inserted at the blunt ended *Hin*dIII site as a blunt ended *Eco*RI fragment. The *Hin*dIII and *Eco*RI sites were blunted by the Klenow fill in reaction. The gB gene was inserted in the same transcriptional orientation as the US2 gene in the parental homology vector. The *Eco*RI fragment may be obtained as a 3.0 KB ILT virus genomic fragment.

HOMOLOGY VECTOR 518-46.B3. The plasmid 518-46.B3 was

constructed for the purpose of inserting foreign DNA into HVT. It contains a unique *Hind*III restriction enzyme site into which foreign DNA may be inserted. When a plasmid containing a foreign DNA insert at the *Hind*III site is used according to the DNA COTRANSFECTION FOR GENERATING RECOMBINANT HERPESVIRUSES or the PROCEDURE FOR GENERATING RECOMBINANT HERPESVIRUS FROM OVERLAPPING SUBGENOMIC FRAGMENTS a virus containing the foreign DNA will result. This plasmid may be constructed utilizing standard recombinant DNA techniques (Maniatis et al, 1982 and Sambrook et al, 1989), by joining three restriction fragments from the following sources. The first fragment is an approximately 1649 base pair *Pvu*I to *Sal*I restriction fragment of pSP64 (Promega). The second fragment is an approximately 1368 base pair *Pvu*I to *Sal*I restriction fragment of pSP65 (Promega). The third fragment is the approximately 3400 base pair *Xho*I to *Xho*I fragment of plasmid 437-47.1.

HOMOLOGY VECTOR 535-70.3. The plasmid 535-70.3 was constructed for the purpose of inserting the MDV gB, and gA genes and the NDV F gene into HVT. The F gene was inserted as a cassette into homology vector 456-17.22 at the *Hind*III site located between the MDV gA and gB genes (see Figure 10A junction B). The F gene is under the control of the HCMV immediate early promoter and followed by the HSV-1 TK poly adenylation signal. The F gene was inserted in the same transcriptional orientation as the US2 gene in the parental homology vector. The cassette may be constructed utilizing standard recombinant DNA techniques (Maniatis *et al*, 1982 and Sambrook *et al*, 1989), by joining restriction fragments from the following sources. The first fragment is an approximately 1191 base pair *Pst*I to *Ava*II restriction sub-fragment of the HCMV genomic *Xba*I E fragment (D.R. Thomsen, *et al*., 1981). The second fragment is an approximately 1812

base pair *Bam*HI to *Pst*I restriction fragment of the full length NDV F cDNA clone (B1 strain). The last fragment is an approximately 784 base pair *Sma*I to *Sma*I restriction sub-fragment of the HSV-1 *Bam*HI restriction fragment Q (McGeoch, *et al.*, 1985).

HOMOLOGY VECTOR 549-24.15. The plasmid 549-24.15 was constructed for the purpose of inserting the MDV gB, and gA genes and the NDV HN and F genes into HVT. The HN and F genes were inserted as a cassette into homolgy vector 456-17.22 at the *Hind*III site located between the MDV gA and gB genes (see Figure 10A junction B). The HN and F genes are under the control of the PRV gpX and HCMV immediate early promoters respectively. The HN and F genes are followed by the PRV gX poly and HSV-1 TK adenylation signals respectively. The cassette may be constructed utilizing standard recombinant DNA techniques (Maniatis *et al*, 1982 and Sambrook *et al*, 1989), by joining restriction fragments from the following sources. The first fragment is an approximately 413 base pair *Sal*I to *Bam*HI restriction sub-fragment of the PRV *Bam*HI fragment #10 (Lomniczi, et al., 1984) The second fragment is an approximately 1811 base pair *Ava*II to *Nae*I restriction fragment of the full length NDV HN cDNA clone (B1 strain). The third fragment is an approximately 754 base pair *Nde*I to *Sal*I restriction sub-fragment of the PRV *Bam*HI restriction fragment #7 (Lomniczi, et al., 1984). The fourth fragment is an approximately 1191 base pair *Pst*I to *Ava*II restriction sub-fragment of the HCMV genomic *Xba*I E fragment (D.R. Thomsen, *et al.*, 1981). The fifth fragment is an approximately 1812 base pair *Bam*HI to *Pst*I restriction fragment of the full length NDV F cDNA clone (B1 strain). The last fragment is an approximately 784 base pair *Sma*I to *Sma*I restriction sub-fragment of the HSV-1 *Bam*HI restriction fragment Q (McGeoch, *et al.*, 1985).

HOMOLOGY VECTOR 549-62.10. The plasmid 549-62.10 was constructed for the purpose of inserting the MDV gB, and gA genes and the NDV HN gene into HVT. The HN gene was inserted as a cassette into homolgy vector 456-17.22 at the *Hind*III site located between the MDV gA and gB genes (see Figure 10A junction B). The HN gene is under the control of the PRV gpX promoter and followed by the PRV gX poly adenylation signal. The HN gene was inserted in the same transcriptional orientation as the US2 gene in the parental homology vector. The cassette may be constructed utilizing standard recombinant DNA techniques (Maniatis *et al*, 1982 and Sambrook *et al*, 1989), by joining restriction fragments from the following sources. The first fragment is an approximately 413 base pair *Sal*I to *Bam*HI restriction sub-fragment of the PRV *Bam*HI fragment #10 (Lomniczi, et al., 1984) The second fragment is an approximately 1811 base pair *Ava*II to *Nae*I restriction fragment of the full length NDV HN cDNA clone (B1 strain). The last fragment is an approximately 754 base pair *Nde*I to *Sal*I restriction sub-fragment of the PRV *Bam*HI restriction fragment #7 (Lomniczi, et al., 1984).

SUBGENOMIC CLONE 550-60.6 Plasmid 550-60.6 was constructed for the purpose of generating recombinant HVT. It contains an approximately 12,300 base pair region of genomic HVT DNA. It may be used in conjunction with other subgenomic clones according to the PROCEDURE FOR GENERATING RECOMBINANT HERPESVIRUS FROM OVERLAPPING SUBGENOMIC FRAGMENTS for the construction of recombinant HVT. This plasmid may be constructed utilizing standard recombinant DNA techniques (Maniatis et al, 1982 and Sambrook et al, 1989), by joining two restriction fragments from the following sources. The first fragment is an approximately 4176 base pair *Eco*RV to *Bam*HI restriction fragment of pBR322. The second fragment is the approximately 12,300 base pair sub-fragment of the *Bam*HI

#2 fragment of HVT (Buckmaster et al., 1988). This fragment was generated in the following manner. Plasmid 437-26.26 was linearized with HindIII and then resected with the ExoIII Mung Bean Deletion Kit (Stratagene). Samples from the 3 and 4 minute reactions were combined and digested with *Bam*HI resulting in a population of fragments containing the desired 12,300 base pair sub-fragment. This population was cloned into the pBR322 fragment and the resulting clones were screened for the appropriate size and restriction map. Fortuitously the resected sub-fragment that generated clone 550-60.6 ended in the nucleotides GG which generated a second *Bam*HI site when ligated to the *Eco*RV site (ATCC) of pBR322. A bacterial strain containing this plasmid has been deposited on March 3, 1993 pursuant to the Budapest Treaty on the International Deposit of Microorganisms for the Purposes of Patent Procedure with the Patent Culture Depository of the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852 U.S.A. under ATCC Accession No. 75429.

HOMOLOGY VECTORS 566-41.5. The plasmid 566-41.5 was constructed for the purpose of inserting the MDV gA, gB and gD genes into HVT. The MDV gD gene was inserted as a *Hin*dIII fragment into the homology vector 456-17.22 at the *Hin*dIII site located between MDV gA and gB (see figure 10). The MDV gene was inserted in the same transcriptional orientation as gA and gB in the parental homology vector. A detailed description of the *Hin*dIII fragment containing the MDV gD gene is shown in figure 11. Note that a herpesvirus polyadenation signal was added to the gD gene cassette. The inserted *Hin*dIII fragment may be constructed utilizing standard recombinant DNA techniques (Maniatis *et al*, 1982 and Sambrook *et al*, 1989), by joining restriction fragments from the following sources with the synthetic DNA sequences indicated in figure 11. Fragment 1 is an approximately 784

base pair *Sma*I to *Sma*I restriction sub-fragment of the HSV-1 *Bam*HI restriction fragment Q (McGeoch *et al.*, 1988). Note that this fragment is oriented such that the polyadenylation sequence (AATAAA) is located closest to junction B. Fragment 2 is an approximately 2177 base pair *Sal*I to *Nco*I sub-fragment of the MDV *Bgl*II 4.2 KB genomic restriction fragment (Ross, *et al.*, 1991).

HOMOLOGY VECTOR 567-72.1D. The plasmid 567-72.1D was constructed for the purpose of inserting the MDV gB, gC and gD genes and the infectious bronchitis virus (IBV) matrix and spike genes into HVT. The IBV genes were inserted as a cassette into homolgy vector 566-41.5 at the unique *Not*I site located upstream of the MDV gD gene (see Figure 11B junction C). The IBV spike and matrix genes are under the control of the HCMV immediate early and PRV gpX promoters respectively. The IBV spike and matrix genes are followed by the HSV-1 TK and PRV gX poly adenylation signals respectively. The IBV genes were inserted in the same transcriptional orientation as the US2 gene in the parental homology vector. The cassette may be constructed utilizing standard recombinant DNA techniques (Maniatis *et al.*, 1982 and Sambrook *et al*, 1989), by joining restriction fragments from the following sources. The first fragment is an approximately 413 base pair *Sal*I to *Bam*HI restriction sub-fragment of the PRV *Bam*HI fragment #10 (Lomniczi, et al., 1984) The second fragment contains amino acids 1 to 223 of the IBV matrix gene. The coding region was obtained from a cDNA clone of the Arkansas strain of IBV. The third fragment is an approximately 754 base pair *Nde*I to *Sal*I restriction sub-fragment of the PRV *Bam*HI restriction fragment #7 (Lomniczi, et al., 1984). The fourth fragment is an approximately 1191 base pair *Pst*I to *Ava*II restriction sub-fragment of the HCMV genomic *Xba*I E fragment (D.R. Thomsen, *et al.*, 1981). The fifth fragment contains amino acids 4 to

1162 of the IBV spike gene. The coding region was obtained from a cDNA clone of the Arkansas strain of IBV. The last fragment is an approximately 784 base pair *Sma*I to *Sma*I restriction sub-fragment of the HSV-1 *Bam*HI restriction fragment Q (McGeoch, *et al.*, 1985).

HOMOLOGY VECTOR 603-57.F1. The plasmid 603-57.F1 was constructed for the purpose of inserting the IBDV VP2 gene into HVT. The IBDV VP2 gene was inserted as a cassette into homolgy vector 603-47.1 at the unique *Hind*III site. The VP2 gene is under the control of the HCMV immediate early promoter and is followed by the HSV-1 TK poly adenylation signal. The VP2 gene was inserted in the same transcriptional orientation as the US2 in the parental homology vector. The cassette may be constructed utilizing standard recombinant DNA techniques (Maniatis *et al*, 1982 and Sambrook *et al*, 1989), by joining restriction fragments from the following sources. The first fragment is an approximately 1191 base pair *Pst*I to *Ava*II restriction sub-fragment of the HCMV genomic *Xba*I E fragment (D.R. Thomsen, *et al.*, 1981). The second fragment is an approximately 1081 base pair *Bcl*I to *Bam*HI restriction sub-fragment of the full length IBDV cDNA clone (see SEQ ID NO:1). Note that the *Bcl*I site was introduced into the cDNA clone directly upstream of the VP2 initiator methionine by converting the sequence CGCAGC to TGATCA. The first and second fragments are oriented such that *Ava*II and *Bcl*I sites are contiguous. The third fragment is an approximately 784 base pair *Sma*I to *Sma*I restriction sub-fragment of the HSV-1 *Bam*HI restriction fragment Q (McGeoch, *et al.*, 1985).

HOMOLOGY VECTOR 633-13.27. The plasmid 633-13.27 was constructed for the purpose of inserting the MDV gB, gA and gD genes and the NDV HN and F genes into HVT. The HN and F genes are under the control of the PRV gpX and HCMV

immediate early promoters respectively. The HN and F genes are followed by the PRV gX poly and HSV-1 TK adenylation signals respectively. All five genes were inserted in the same transcriptional orientation as the US2 gene in the parental homology vector. The genes were inserted in the following order MDV gA, NDV HN, NDV F,MDV gD, and MDV gB.

HOMOLOGY VECTOR 634-29.16. The plasmid 634-29.16 was constructed for the purpose of inserting the ILT virus gB and gD genes into HVT. The *lacZ* marker gene followed by the ILT gB and gD genes inserted as a cassette into the homology vector 172-29.31 at the unique *Xho*I site. The cassette may be constructed utilizing standard recombinant DNA techniques (Maniatis *et al*, 1982 and Sambrook *et al*, 1989), by joining restriction fragments from the following sources. The first fragment is an approximately 4229 base pair *Sal*I to *Sal*I restriction fragment derived from the *lacZ* marker gene described above and shown in figure 7. The second fragment is an approximately 2060 base pair *Eco*RI to *Bcl*I restriction sub-fragment of the ILT *Kpn*I genomic restriction fragment #8 (10.6 KB). The third fragment is an approximately 754 base pair *Nde*I to *Sal*I restriction sub-fragment of the PRV *Bam*HI restriction fragment #7 (Lomniczi *et al.*, 1984). Note that the second and third fragments are oriented such that *Bcl*I and *Nde*I sites are contiguous. The fourth fragment is the 3.0 KB ILT virus genomic *Eco*RI fragment containing the gB gene. All three genes are in the same transcriptional orientation as the UL43 gene.

EXAMPLES

EXAMPLE 1

S-HVT-001

S-HVT-001 is a herpesvirus of turkeys (HVT) that contains the *E. coli* β-galactosidase gene inserted into the unique long region of the HVT genome. The restriction enzyme map of HVT has been published (T. Igarashi, *et al.*, 1985). This information was used as a starting point to engineer the insertion of foreign genes into HVT. The *Bam*HI restriction map of HVT is shown in Figure 1A. From this data, several different regions of HVT DNA into which insertions of foreign genes could be made were targeted. The foreign gene chosen for insertion was the *E. coli* β-galactosidase (*lacZ*) gene , which we have used in PRV. The promoter was the PRV gpX promoter. The *lacZ* gene was inserted into the unique long region of HVT, specifically into the *Xho*I site in the *Bam*HI #16 (3329bp) fragment, and was shown to be expressed in an HVT recombinant by the formation of blue plaques using the substrate Bluogal® (Bethesda Research Labs). Similarly, the *lacZ* gene has been inserted into the *Sal*I site in the repeat region contained within the *Bam*HI #19 (900 bp) fragment.

These experiments show that HVT is amenable to the procedures described within this application for the insertion and expression of foreign genes in herpesviruses. In particular, two sites for insertion of foreign DNA have been identified (Figs. 1B and 1C).

EXAMPLE 2

S-HVT-003

S-HVT-003 is a herpesvirus of turkeys (HVT) that contains the *E. coli* β-galactosidase (*lacZ*) gene and the infectious bursal disease virus (IBDV) strain S40747 large segment of RNA (as a cDNA copy) inserted into the unique long region of the HVT genome. This IBDV DNA contains one open reading frame that encodes three proteins (5'VP2-VP4-VP3 3'), two of which are antigens to provide protection against IBDV infections of chickens. Expression of the genes for both β-galactosidase and the IBDV polyprotein are under the control of the pseudorabies virus (PRV) gpX gene promoter. S-HVT-003, deposited under ATCC Accession No. VR 2178, was made by homologous recombination.

The IBDV genes were cloned by the cDNA CLONING PROCEDURE. Clones representing the genome of IBDV were screened by SOUTHERN BLOTTING OF DNA procedure against blots containing authentic IBDV RNA. Positive clones were then characterized by restriction mapping to identify groups of clones. Two such clones were identified, that together were found to represent the entire coding region of the IBDV large segment of RNA (3.3 kb dsRNA). One cDNA clone (2-84) contained an approximately 2500 base pair fragment representing the first half of the IBDV gene. The second clone (2-40) contained an approximately 2000 base pair fragment representing the distal half of the IBDV gene. Plasmid 2-84/2-40, representing the entire IBDV gene, was constructed by joining clone 2-84 and 2-40 at a unique *Pvu*II site present in the overlapping sequences. The IBDV genome can be obtained from plasmid 2-84/2-40 as an approximately 3400 base pair *Sma*I to *Hpa*I fragment. Confirmation of the nature of the proteins encoded by the IBDV gene was obtained by

expressing the clone (2-84/2-40) in *E. coli* and detecting VP3 antigen using antiserum made against purified IBDV capsid proteins on Western blots. Applicants' sequence of the large DNA segment that encodes the IBDV antigens is given seq ID # 1. This sequence shows one open reading frame that will henceforth be referred to as the IBDV gene. The sequence of an Australian IBDV strain has been published which bears close homology to applicants' sequence (Hudson *et al*,1986). Comparison of the amino acid differences between the two viruses revealed 29 amino acid changes within the 1012 amino acid coding region. There were only 3 amino acid differences deduced for VP4 and only 8 in VP3. In contrast, VP2 contained 18 amino acid changes, 14 of which were clustered between amino acids 139 to 332.

For insertion into the genome of HVT, the coding region for the IBDV gene was cloned between the PRV gpX promoter and the HSV TK poly-A signal sequence, creating plasmid 191-23. To aid in the identification of HVT recombinants made by homologous recombination containing the IBDV gene the gpX promoted IBDV fragment from plasmid 191-23 was inserted behind (in tandem to) a *lacZ* gene controlled by a gpX promoter. The resultant plasmid, 191-47, contains the *E.coli lacZ* gene and the IBDV gene under the control of individual PRV gpX promoters. In constructing plasmid 191-47, various DNA fragments were joined by recombinant DNA techniques using either naturally occurring restriction sites or synthetic linker DNA. Details concerning the construction of these genes contained in plasmid 191-47 can be seen in figure 2. The first segment of DNA (segment 1, figure 2) contains the gpX promoter region including the residues encoding the first seven amino acids of the gpX gene, and was derived from a subclone of the PRV *Bam*HI #10 fragment as an approximately 800 base pair *Sal*I to *Bam*HI fragment. The second segment of DNA (segment 2, figure 2)

contains the *E. coli* β-galactosidase coding region from amino acid 10 to amino acid 1024 and was derived from the plasmid pJF751 (obtained from Jim Hoch, Scripps Clinic and Research Foundation) as an approximately 3300 base pair *Bam*HI to *Bal*I fragment followed by an approximately 40 base pair Ava I to Sma I fragment. The third segment of DNA (segment 3, figure 2) contains the gpX poly A signal sequence and was derived from a subclone of the PRV *Bam*HI #7 fragment as an approximately 700 base pair *Nde*I to *Stu*I fragment. Segment three was joined to segment two by ligating the *Nde*I end which had been filled in according to the POLYMERASE FILL-IN REACTION, to the *Sma*I site. The fourth segment of DNA (segment 4 figure 2) contains the gpX promoter (TATA box and cap site) and was derived from a subclone of the PRV *Bam*HI #10 fragment as an approximately 330 base pair *Nae*I to *Alu*I fragment. Additionally, segment four contains approximately 36 base pairs of HSV TK 5'untranslated leader sequence as a *Pst*I to *Bgl*II fragment in which the *Pst*I site has been joined to the *Alu*I site through the use of a synthetic DNA linker (McKnight and Kingbury, 1982). DNA segments four through six were inserted as a unit into the unique *Kpn* I site of segment three which is located 3' of the gpX poly A signal sequence. The fifth segment of DNA (segment 5, figure 2) contains the entire coding region of the IBDV large segment of RNA (cDNA clone) as an approximately 3400 base pair *Sma*I to *Hpa*I fragment. The *Sma*I site of segment five was fused to the *Bgl*II site of segment four which had been filled in according to the POLYMERASE FILL IN REACTION Expression of the IBDV gene (5'VP2-VP4-VP3 3'), is under the control of the gpX promoter (segment 4), but utilizes its own natural start and stop codons. The sixth segment of DNA (segment 6, figure 2) contains the HSV TK poly-A signal sequence as an

approximately 800 base pair *Sma*I fragment (obtained from Bernard Roizman, Univ. of Chicago). The *Hpa*I site of segment five was fused to the *Sma*I site of segment six through the use of a synthetic DNA liner.

In summary, the construct used to create S-HVT-003 (plasmid 191-47) contains (5' to 3') the PRV promoter, the gpX TATA box, the gpX cap site, the first seven amino acids of gpX, the *E. coli* β-galactosidase (*lacZ*) gene, the PRV poly-A signal sequence, the PRV gpX promoter, the gpX TATA box, the gpX cap site, a fusion within the gpX untranslated 5' leader to the IBDV gene, IBDV start codon, a fusion within the IBDV untranslated 3' end to HSV TK untranslated 3' end, and the TK poly-A signal sequence. The cassette containing these genes was engineered such that it was flanked by two *Eco*RI restriction endonuclease sites. As a result, an approximately 9100 base pair fragment containing both *lacZ* gene and the IBDV gene can be obtained by digestion with *Eco*RI. Henceforth, the 9161 base pair *Eco*RI fragment will be referred to as the IBDV/*lacZ* cassette. The following procedures were used to construct S-HVT-003 by homologous recombination. The IBDV/*lacZ* cassette was inserted into the unique *Xho*I site present within a subclone of the HVT *Bam*HI #16 fragment. To achieve this, the *Xho*I site was first changed to an *Eco*RI site through the use of an *Eco*RI linker. This site had previously been shown to be nonessential in HVT by the insertion of *lacZ* (S-HVT-001). It was also shown that the flanking homology regions in *Bam*HI #16 were efficient in homologous recombination. Shown in Figure 3, the genomic location of the *Bam*HI #16 fragment maps within the unique long region of HVT. The complete nucleotide sequence of the approximately 3329 base pair *Bam*HI #16 fragment is presented as seq ID #3. HVT DNA was prepared by the PREPARATION OF HERPESVIRUS DNA procedure. Cotransfections of HVT DNA and plasmid DNA into primary

chick embryo fibroblast (CEF) cells were done according to the DNA TRANSFECTION FOR GENERATING RECOMBINANT HERPESVIRUS. The recombinant virus resulting from the cotransfection stock was purified by three successive rounds of plaque purification using the BLUOGAL SCREEN FOR RECOMBINANT HERPESVIRUS procedure. When 100% of the plaques were blue, the DNA was analyzed for the presence of the IBDV gene by the SOUTHERN BLOTTING OF DNA procedure. Southern blots, probing *Eco*RI digested S-HVT-003 DNA with an IBDV specific nick translated probe (plasmid [illegible]), confirmed the presence of the 9100 base pair *Eco*RI fragment. This result confirmed that S-HVT-003 contained both the *lac*Z gene and the IBDV gene incorporated into its genome. Additional Southern blots, using a probe specific for *Bam*HI #16, confirmed that the homologous recombination occurred at the appropriate position in *Bam*HI #16 and that no deletions were created. No differences in the growth of S-HVT-003 compared to wild type virus (S-HVT-000) were observed *in vitro*.

Expression of IBDV specific proteins from S-HVT-003 were assayed *in vitro* using the WESTERN BLOTTING PROCEDURE. Cellular lysates were prepared as described in PREPARATION OF HERPESVIRUS CELL LYSATES. Briefly, the proteins contained in the cellular lysates of S-HVT-003 were separated by polyacrylamide gel electrophoresis, transferred to nitrocellulose, and probed with either an antiserum made against denatured purified IBDV capsid proteins or antiserum made against a synthetic peptide corresponding to a predicted imuno dominant region of the IBDV 40 kd (VP2) capsid protein. The filters were washed and treated with [^{125}I] protein A to detect the position of the bound antibodies. Figure 4 shows the results obtained using the antiserum made against denatured purified IBDV capsid proteins, which have been shown by the applicants to react primarily with VP3 (32 kd protein). As seen, S-HVT-003

produces a protein which is immunologically indistinguishable from the authentic VP3 protein from intact IBDV virions. Moreover, the polyprotein appears to be processed correctly, producing a VP3 species that comigrates with the authentic VP3 protein. Recent evidence using an Australian IBDV stain indicates that VP4 is involved in the processing of the precursor polyprotein into mature VP2 and VP3 protein species (Jagadish, *et al.*, 1988). Figure 5 shows the results obtained using a rabbit antiserum raised against a synthetic peptide that is homologous to a 14 amino acid region of the IBDV VP2 (40 kd) capsid protein. As seen, S-HVT-003 produces a protein that is immunologically indistinguishable from the authentic viral VP2 protein. In addition, the VP2 protein produced from S-HVT-003 comigrates with the 40 kd species of VP2 isolated from intact IBDV virions. This species represents a major component of infectious (complete) viral particles.

In summary, analysis of the expression of IBDV specific proteins from S-HVT-003 has shown that the polyprotein is processed in CEF cell culture, producing proteins of the appropriate size that react to immunological reagents specific for either VP2 or VP3 proteins on Western blots.

The following set of experiments was carried out in chickens to analyze the *in vivo* expression of the IBDV genes contained within S-HVT-003 as determined by seroconversion data, serum neutralization results, and protection from IBDV challenge.

The first experiment was designed to show the seroconversion of chickens to IBDV upon being vaccinated with S-HVT-003. Eleven 11-week-old chickens, seronegative to HVT and IBDV were obtained from SPAFAS Inc. Six birds were vaccinated subcutaneously in the abdominal region with

0.5 ml of a cellular suspension of CEF cells containing S-HVT-003 (40,000 PFU/ml). Serum samples were obtained every seven days for eight weeks for all birds in this study. On day 28 (4th week), three of these birds received a boost of S-HVT-003, while the other three birds received 0.5 ml of an inactivated IBDV vaccine inoculated subcutaneously in the cervical region. Three additional birds were given only the inactivated vaccine on day 28. Two birds served as contact controls and received no vaccinations. On day 56, all birds were sacrificed and necropsied. Table 1 show the results of the serum neutralization assay against IBDV. No detectable SN activity was observed in the birds given only S-HVT-003. Additionally, only one of the three birds that were given only the inactivated vaccine demonstrated low but detectable SN activity. SN titers were also detected in one of the three birds that received the S-HVT-003 followed by the inactivated IBDV vaccine boost; these titers were at a much higher level than with the inactivated IBDV vaccine alone. These results suggest that S-HVT-003 is priming the chicken for a secondary response against IBDV. *In vitro* analysis of the serum samples by WESTERN BLOTTING confirmed the seroconversion of the chickens to IBDV upon vaccination with S-HVT-003 both prior to and after boosts administered on day 28.

TABLE 1

Vaccine Group	Bird No.	DAY 28	31	35	38	42	49
HVT-003	265	<2	<2	<2	<2	<2	<2
HVT-003	266	<2	<2	<2	<2	<2	<2
	267	<2	<2	<2	<2	<2	<2
HVT-003	260	<2	<2	<2	<2	<2	<2
IBDV[a]	264	<2	<2	<2	1:64	1:256	1:512
	269	<2	<2	<2	<2	<2	<2
C	261	<2	<2	<2	<2	<2	<2
IBDV[a]	262	<2	<2	<2	<2	1:4	1:4
	263	<2	<2	<2	<2	<2	<2
C	270	<2	<2	<2	<2	<2	<2
	271	<2	<2	<2	<2	<2	<2

[a] Commercial

In the second experiment, twenty five 1-day old SPF chicks were vaccinated with S-HVT-003 (20 with 0.2ml subcutaneously and 5 by bilateral eyedrop). Twenty chicks were kept as controls. On days four and seven postinfection, five vaccinates and two control birds were bled, sacrificed and their spleens removed for virus isolation. Spleen cell suspensions were made by standard method, and ~1 x 10^6 cells in 3 ml of chick embryo fibroblast (CEF) growth media were inoculated directly onto secondary cells. Cultures were incubated for 6-7 days and then scored for cytopathic effects (CPE) as determined by observing cell morphology. The cultures were passed a second time, and again scored for CPE. The results are shown in Table 2. All nonvaccinated control birds remained negative for HVT for both day 4 and 7 spleen cell isolations. Four out of the five birds

vaccinated with S-HVT-003 were positive for HVT at day 4 for both the first and second passages. One bird did not produce virus, this may represent a vaccination failure. Five out of five birds were positive for HVT on day 7 at both passage one and two. Overall, the vector recovery experiment demonstrates that S-HVT-003 replicates as well as wild type HVT virus *in vivo* and that insertion of the IBDV/*lacZ* cassette into the *Xho*I site of *Bam*HI #16 does not result in detectable attenuation of virus. Subsequent experiments examining the recovered virus by the DIFCO SCREEN FOR RECOMBINANT HERPESVIRUS procedure confirmed the *in vivo* stability of S-HVT-003, by demonstrating β-galactosidase expression in 100% of the viruses.

TABLE 2

	Harvest Date			
	Day 4		Day 7	
Sample	P1	P2	P1	P2
N 1	-	-		
N 2	-	-		
N 3			-	-
N 4			-	-
T 1	-	-		
T 2	2+	2+		
T 3	2+	2+		
T 4		4+		
T 5	3+	3+		
T 6			2+	contaminated
T 7			+	5+
T 8			+	5+
T 8			+	5+
T 9			+	5+
T10			+	5+

N = control, T = vaccinated

CPE ranged from negative (-) to 5+

At days 0, 4, 7, 14, 21, and 27 postinfection, blood samples were obtained from the rest of the chickens for determining serum ELISA titers against IBDV and HVT antigens as well as for virus neutralizing tests against IBDV. Additionally, at 21 days postinfection five control and fourteen vaccinated chicks were challenged with virulent IBDV by bi-lateral eyedrop ($10^{3.8}EID_{50}$). All birds were sacrificed 6-days post challenge and bursa to body weight ratios were calculated. A summary of the results is shown in tables X and XI, respectively. As presented in Table 3, no antibodies were detected against HVT antigens by ELISA prior to 21-27 days post vaccination. In chickens, the immune response during the first two weeks post hatch is both immature and parentally suppressed, and therefore these results are not totally unexpected. In contrast, IBDV ELISA's were negative up to day 21 postvaccination, and were only detectable after

challenge on day 27. The ELISA levels seen on day 27 postvaccination indicate a primary response to IBDV. Table 3I comparing the Bursa-to-Body weight ratios for challenged controls and vaccinated/challenged groups show no significant differences. Vaccination with S-HVT-003 under these conditions did not prevent infection of the vaccinated birds by IBDV challenge, as indicated by the death of four vaccinated birds following challenge.

TABLE 3

Sample Group		ELISA HVT	ELISA IBDV	VN IBDV
C-0	(n=3)	0	0	<100
C-4	(n=2)	0	0	nd
T-4	(n=5)	0	0	nd
C-7	(n=2)	0	0	<100
T-7	(n=5)	0	0	<100
C-14	(n=5)	0	0	nd
T-14	(n=14)	0	0	<100
C-21	(n=5)	0	0	nd
T-21	(n=14)	1	0	<100
C-27	(n=5)	0	0	nd
CC-27	(n=5)	0	5	nd
CT-27	(n-10)	3.2	2	nd

C=control, T=vaccinated, CC=challenged control, CT=Challenged & vaccinated. ELISA titers are GMTs and they range from 0-9.

TABLE 4

Sample Group	Body wt.	Bursa wt.	BBR
Con.(n=5)	258.8	1.5088	0.0058
Chall.	209	0.6502	0.0031
Con.(n=5) Chall. Treated (n=10)	215.5	0.5944	0.0027

Values are mean values. Body weights are different in control group because challenged birds did not feed well. Four challenged-treated birds died.

A third experiment was conducted repeating Experiment 2 but using immunologically responsive chicks (3 weeks of age). Six three week old SPF leghorn chickens were vaccinated intraperitoneally with 0.2ml of S-HVT-003 (one drop in each eye). Serum samples were obtained every seven days for six-weeks and the birds were challenged with the virulent USDA standard challenge IBDV virus on day 43 postvaccination.

Six days post challenge, the control, vaccinated-challenged, and challenged groups were sacrificed and bursas were harvested for probing with anti-IBDV monoclonal antibodies (MAB) (provided by Dr. David Snyder, Virginia-Maryland Regional College of Veterinary Medicine). Bursal homogenates were prepared by mixing 1 ml of 0.5% NP40 with one bursa. Bursa were then ground and briefly sonicated. Supernatants from the homogenates were reacted with the R63 MAB which had been affixed to 96-well Elisa plates via a protein A linkage. After incubation, a biotin labeled preparation of the R63 MAB was added. After washing, an avidin-horse radish peroxidase conjugate was added and incubated. Tests were developed with Tris-malcate buffer (TMB) + H_2O_2 substrate. The test results are presented in Table 5. The data show the presence of high levels of IBDV antigen in all bursa in the vaccinate-challenged group and in the challenged group. No IBDV antigen was detected in the controls. IBDV specific antigen could be detected at dilutions of over 1/1000, and there does not appear to be differences between vaccinated and non-vaccinated challenged groups. HVT titers as determined by ELISA were first detectable at day 7 in four out of the six birds vaccinated. By day 14, six out of six vaccinated birds showed titers to HVT. All six birds continued to show HVT titers throughout the experiment. No IBDV SN titers were seen prior to the challenge. In contrast, analysis of these same serum samples by the WESTERN BLOTTING procedure demonstrated the seroconversion of chickens vaccinated with S-HVT-003 to IBDV prior to administration of the virus challenge. The level of response, however, remains small unless boosted by challenge. Comparison between the vaccinated/challenged and challenged only groups clearly demonstrates that the level of reactivity by Western blots is much higher in the vaccinated/challenged group. These results show that S-HVT-003 is seroconverting vaccinated birds to IBDV, and suggest

that the level of IBDV specific expression are not high enough to induce a neutralizing response in the birds.

S-HVT-003 shows the merit of the vaccine approach the applicants have invented. HVT has been engineered to simultaneously express the foreign antigens (β-galactosidase and IBDV antigens) that are recognized in the host by an immune response directed to these proteins.

TABLE 5

Serology: Herpes/IBDV ELISA titer

		Bleed Date							
	Bird#	11/3	11/10	11/14	11/24	12/1	12/8	12/15	12/22
	Vaccinated Challenged								
	221	0/0	7/0	5/0	6/0	5/0	5/0	5/0	3/3
	41	0/0	4/0	4/0	1/0	1/0	1/0	1/0	1/3
	42	0/0	3/0	2/0	1/0	5/0	5/0	5/0	3/2
	43	0/0	0/0	5/0	5/0	5/0	5/0	3/0	3/2
	44	0/0	1/0	5/0	1/0	2/0	1/0	1/0	2/4
	45	0/0	0/0	1/0	1/0	1/0	1/0	1/0	1/3
	Control								
	28	0/0							0/0
	35	0/0							0/0
	73	0/0							0/0
	75	0/0							0/0
	Challenged only								
	40	0/0							0/3
	74	0/0							0/5
	39	0/0							0/3
	72	0/0							0/3

Maximum titer level is 9

Example 3

S-HVT-004

S-HVT-004 is a recombinant herpesvirus of turkeys that contains the Marek's disease virus (MDV) glycoprotein A (gpA) gene inserted into the long unique region, and the β-galactosidase (*lacZ*) gene also inserted in the long unique region. The MDV antigen is more likely to elicit the proper antigentic response than the HVT equivalent antigen.

The MDV gpA gene was cloned by standard DNA cloning gpA procedures. An *Eco*RI restriction fragment had been reported to contain the MDV gpA gene (Isfort *et al.*, 1984) and this fragment was identified by size in the DNA clones. The region of the DNA reported to contain the gpA gene was sequenced by applicants and found to contain a glycoprotein gene as expected. The DNA from this gene was used to find the corresponding gene in HVT by the SOUTHERN BLOTTING OF DNA procedure, and a gene in HVT was identified that contained a very similar sequence. This gene is the same gene previously called gpA (Isfort *et al.*, 1984).

For insertion into the genome of HVT, the MDV gpA gene was used intact because it would have good herpesvirus signal sequences already. The *lacZ* gene was inserted into the *Xho*I fragment in *Bam*HI fragment #16, and the MDV gpA gene was inserted behind *lacZ* as shown in Figure 6A and 6B. Flanking regions in *Bam*HI #16 were used for the homologous recombination. HVT DNA and plasmid DNA were co-transfected according to the DNA TRANSPECTION FOR GENERATING RECOMBINANT HERPESVIRUS procedure into primary chick embryo fibroblast (CEF) cells. The virus from the transfection stock was purified by successive plaque purifications using the BLUOGAL SCREEN FOR RECOMBINANT HERPESVIRUS procedure. At

the end of this procedure, when 100% of the plaques were blue, the DNA was analyzed for the presence of the MDV gpA gene. S-HVT-004 is a recombinant virus that contains both the β-galactosidase gene and the MDV gpA gene incorporated into the genome.

Figure 6C shows the structure of S-HVT-004.

Example 4

NEWCASTLE DISEASE VIRUS

Newcastle disease virus (NDV) is closely related to PI-3 in overall structure. We have engineered the hemagglutinin (HN) and fusion (F) genes of PI-3 for expression in IBR (ref). Similarly we have cloned the hemagglutinin (HN) and fusion (F) genes from NDV for use in the herpesvirus delivery system (Herpesvirus of turkeys, HVT).

The procedures that we have utilized for construction of herpesvirus control sequences for expression have been applied to NDV.

INFECTIOUS BRONCHITIS VIRUS

Infectious bronchitis virus (IBV) is a virus of chickens closely related in overall structure to TGE. We have engineered the major neutralizing antigen of TGE for expression in PRV (ref). Similarly we have cloned the major neutralizing antigens from three strains of IBV: Massachusetts (SEQ ID NO: 14), Connecticut (SEQ ID NO: 16), and Arkansas-99 (SEQ ID NO: 18) for use in a herpesvirus delivery system (HVT).

The procedures that we have utilized for the construction of herpesvirus control sequences for expression have been applied to IBV.

EXAMPLE 5

S-HVT-045

S-HVT-045 is a recombinant herpesvirus of turkeys that contains the Marek's disease virus (MDV) glycoprotein B (gpB) gene inserted into the short unique region. The MDV antigen is more likely to elicit the proper antigenic response than the HVT equivalent antigen. S-HVT-045 has been deposited on October 15, 1992 pursuant to the Budapest Treaty on the International Deposit of Microorganisms for the Purposes of Patent Procedure with the Patent Culture Depository of the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852 U.S.A. under ATCC Accession No. VR 2383.

The MDV gpB gene was cloned by standard DNA cloning procedures. The MDV gpB gene was localized to a 3.9 kb *Eco*RI-*Sal*I fragment using an oligonucleotide probe based on the HSV gB sequence in a region found to be conserved among known herpesvirus gB genes. The restriction map 3.9 kb *Eco*RI-*Sal*I fragment is similar to the published map (Ross et al., 1989).

For insertion into the HVT genome, the MDV gpB was used intact because it would have good herpesvirus signal sequences already. The MDV gpB gene was inserted into a cloned 17.15 kb *Bam*HI-*Eco*RI fragment derived from the HVT *Bam*HI #1 fragment. The site used for insertion was the *Stu*I site within HVT US2, previously utilized for the construction of S-HVT-012. The site was initially altered by insertion of a unique *Hind*III linker, and the MDV gpB gene was inserted by standard DNA cloning procedures. Flanking regions in the 17.15 kb *Bam*HI-*Eco*RI fragment were used, together with the remaining cloned HVT fragments using

the PROCEDURE FOR GENERATING RECOMBINANT HERPESVIRUSES FROM OVERLAPPING SUBGENOMIC FRAGMENTS. The virus obtained from the transfection stock was plaque purified and the DNA was analyzed for the presence of the MDV gpB gene. S-HVT-045 is a recombinant virus that contains the MDV gpB gene incorporated into the genome at the *Stu*I site in HVT US2 gene.

TESTING OF RECOMBINANT S-HVT-045

Two studies were conducted to demonstrate the effectiveness of these recombinant HVT/MDV viruses in protecting against challenge with virulent Marek's disease virus. In Study A, one-day-old specific pathogen free (SPF) chicks were vaccinated with either S-HVT-045 or S-HVT-046. Seven days post-vaccination, vaccinated chicks, and non-vaccinated, control chicks were challenged with the highly virulent MD-5 strain of Marek's disease virus. Following a 6-week post-challenge observation period for clinical signs typical of Marek's disease, all chicks were necropsied and examined for lesions diagnostic of Marek's disease. The results, in Table 6, show that both recombinant viruses gave complete protection against a challenge that caused Marek's disease in 90% of non-vaccinated control chicks.

In a second study, one-day-old chicks were vaccinated either with S-HVT-045 or S-HVT-047. A third group of chicks were vaccinated with a USDA-licensed, conventional vaccine comprised of HVT and SB-1 viruses. Five days post-vaccination, the vaccinated chicks and a group of non-vaccinated, control chicks were challenged with virulent Marek's virus, strain RB1B. The chicks were observed for 8 weeks for clinical signs of Marek's disease, then necropsied and observed for Marek's lesions. This study demonstrated the ability of HVT-045 and HVT-047 to provide 100%

protection against challenge (Table 1). The commercial vaccine gave 96% protection, and 79% of the non-vaccinated chicks developed Marek's disease.

TABLE 6 EFFICACY OF RECOMBINANT HVT/MDV VIRUSES TO PROTECT SUSCEPTIBLE CHICKS AGAINST VIRULENT MAREK'S DISEASE VIRUS

	Marek's Protection	
Vaccine Group	MD-5 Challenge	RB1B Challenge
S-HVT-045	20/20	24/24
S-HVT-046	20/20	Not Tested
S-HVT-047	Not Tested	24/24
HVT*	Not Tested	24/25
Controls	2/20	5/24

* Commercial

Example 6

S-HVT-012

S-HVT-012 is a recombinant herpesvirus of turkeys that contains the *E. coli* β-galactosidase (*lacZ*) gene inserted into the short unique region. The *lacZ* gene was used to determine the viability of this insertion site in HVT [ATCC F-126 ("Calnek")]. S-HVT-012 has been deposited on October 15, 1992 pursuant to the Budapest Treaty on the International Deposit of Microorganisms for the Purposes of Patent Procedure on with the Patent Culture Depository of the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852 U.S.A. under ATCC Accession No. VR 2382.

For insertion into the genome of HVT, the β-galactosidase gene was introduced into the unique *Stu*I site of the cloned *Eco*RI fragment #7 of HVT, i.e., the fragment containing the *Stu*I site within the US2 gene of HVT (as described in Methods and Materials). Flanking regions of *Eco*RI fragment #7 were used for homologous recombination. HVT DNA and plasmid DNA were co-transfected according to the DNA TRANSFECTION FOR GENERATING RECOMBINANT VIRUS procedure into primary chick embryo fibroblast (CEF) cells. A blue virus obtained from the transfection stock was purified by successive plaque purifications using the BLUOGAL SCREEN FOR RECOMBINANT HERPESVIRUS procedure. At the end of this procedure, when 100% of the plaques were blue, the DNA was analyzed for the presence of the *lacZ* gene. S-HVT-012 is a recombinant virus that contains the *lacZ* gene incorporated into the genome at the *Stu*I site within the US2 gene of HVT.

TESTING OF RECOMBINANT S-HVT-012

S-HVT-012 may be formulated as a vaccine in the same manner as S-HVT-045. When administered to chickens, such a vaccine provides protection against Marek's disease virus.

Example 7

Sites for Insertion of Foreign DNA into HVT

In order to define appropriate insertion sites, a library of HVT *Bam*HI and *Eco*RI restriction fragments was generated. Several of these restriction fragments (*Bam*HI fragments #16 and #13, and *Eco*RI fragments #6, #7, and #9 (see figure 1)) were subjected to restriction mapping analysis. One unique restriction site was identified in each fragment as a potential insertion site. These sites included *Xho*I in *Bam*HI fragments #13 and #16, and *Eco*RI fragment #9 and *Sal*I in *Eco*RI fragment #6 and *Stu*I in *Eco*RI fragment #7. A β-galactosidase (*lacZ*) marker gene was inserted in each of the potential sites. A plasmid containing such a foreign DNA insert may be used according to the DNA COTRANSFECTION FOR GENERATING RECOMBINANT HERPESVIRUSES to CONSTRUCT a HVT containing the foreign DNA. For this procedure to be successful it is important that the insertion site be in a region non-essential to the replication of the HVT and that the site be flanked with HVT DNA appropriate for mediating homologous recombination between virus and plasmid DNAs. The plasmids containing the *lacZ* marker gene were utilized in the DNA COTRANSFECTION FOR GENERATING RECOMBINANT HERPESVIRUSES. The generation of recombinant virus was determined by the BLUOGAL SCREEN FOR RECOMBINANT HERPESVIRUS. Three of the five sites were successfully used to generate a recombinant virus. In each case the resulting virus was easily purified to 100%, clearly defining an appropriate site for the insertion of foreign DNA. The three homology vectors used to define these sites are described below.

Example 7A

Homology Vector 172-29.31

The homology vector 172-29.31 contains the HVT *Bam*HI #16 fragment and is useful for the insertion of foreign DNA into HVT. Plasmid 172-29.31 contains a unique *Xho*I restriction site into which foreign DNA may be cloned. We have demonstrated that the *Xho*I site in homology vector 172-29.31 may be used to insert foreign DNA into HVT by the construction of at least three recombinant HVT (see examples 1-3).

The homology vector 172-29.31 was further characterized by DNA sequence analysis. The complete sequences of the *Bam*HI #16 fragment was determined. Approximately 2092 base pairs of the adjacent *Bam*HI #13 fragment was also determined (see SEQ ID 3). This sequence indicates that the open reading frame coding for HVT glycoprotein A (gA) spans the *Bam*HI #16 - *Bam*HI #13 junction. The HVT gA gene is homologous to the HSV-1 glycoprotein C (gC). The *Xho*I site interrupts an ORF which lies directly upstream of the HVT gA gene. This ORF shows amino acid sequence homology to the PRV p43 and the VZV gene 15. The PRV and VZV genes are the homologues of HSV-1 UL43. Therefore we have designated this ORF as HVT UL43. It should be noted that the HVT UL43 does not exhibit direct homology to HSV-1 UL43. Although HVT UL43 is located upstream of the HVT gC homologue it is encoded on the same DNA strand as HVT gA, where as the HSV-1 UL43 is on the opposite strand relative to HSV-1 gC. The *Xho*I site interrupts UL43 at approximately amino acid 6, suggesting that the UL43 gene is non-essential for HVT replication.

Example 7B

Homology Vector 435-47.R17

The homology vector 435-47.R17 contains the HVT *Eco*RI #7 fragment and is useful for the insertion of foreign DNA into HVT. Plasmid 435-47.R17 contains a unique *Hind*III restriction site into which foreign DNA may be cloned. The *Hind*III restriction site in plasmid results from the insertion of a *Hind*III linker into the naturally occurring *Stu*I site of *Eco*RI fragment #7. We have demonstrated that the *Hind*III site in homology vector 435-47.R17 may be used to insert foreign DNA into HVT by the construction of at least 25 recombinant HVT.

DNA sequence analysis at the *Stu*I indicated that this fragment contains open reading frames coding for US10, US2, and US3. The *Stu*I site interrupts US2 at approximately amino acid 124, suggesting that the US2 gene is non-essential for HVT replication.

Example 7C

Homology Vector 172-63.1

The homology vector 172-63.1 contains the HVT *Eco*RI #9 fragment and is useful for the insertion of foreign DNA into HVT. Plasmid 172-63.1 contains a unique *Xho*I restriction site into which foreign DNA may be cloned. We have demonstrated that the *Xho*I site in homology vector 172-63.1 may be used to insert foreign DNA into HVT by the construction of S-HVT-014 (see example 8).

Example 8

S-HVT-014

S-HVT-014 is a recombinant herpesvirus of turkeys that contains the *E. coli* β-galactosidase (*lacZ*) gene inserted into the long unique region. The *lacZ* gene was used to determine the viability of this insertion site in HVT [ATCC F-126 ("Calnek")].

For insertion into the genome of HVT, the β-galactosidase gene was introduced into the unique *Xho*I site of the cloned *Eco*RI fragment #9 (as described in Methods and Materials). Flanking regions of *Eco*RI fragment #8 were used for homologous recombination. HVT DNA and plasmid DNA were co-transfected according to the DNA TRANSFECTION FOR GENERATING RECOMBINANT VIRUS procedure into primary chick embryo fibroblast (CEF) cells. A blue virus obtained from the transfection stock was purified by successive plaque purifications using the BLUOGAL SCREEN FOR RECOMBINANT HERPESVIRUS procedure. At the end of this procedure when 100% of the plaques were blue. S-HVT-014 is a recombinant virus that contains the *lacZ* gene incorporated into the genome at the *Xho*I site within the *Eco*RI #9 fragment of HVT.

TESTING OF RECOMBINANT S-HVT-014

S-HVT-014 may be formulated as a vaccine in the same manner as S-HVT-045. When administered to chickens, such a vaccine provides protection against Marek's disease virus.

Example 9

S-HVT-005

S-HVT-005 is a recombinant herpesvirus of turkeys that contains the *E. coli* β-galactosidase (*lacZ*) gene inserted into the long unique region. The *lacZ* gene was used to determine the viability of this insertion site in HVT [ATCC F-126 ("Calnek")].

For insertion into the genome of HVT, the β-galactosidase gene was introduced into an approximately 1300 base pair deletion of the *Xho*I #9 fragment of HVT. The deletion which lies between the unique *Mlu*I and *Eco*RV sites removes the complete coding region of the HVT gA gene (see SEQ ID 3). Flanking regions of *Xho*I fragment #9 were used for homologous recombination. HVT DNA and plasmid DNA were co-transfected according to the DNA TRANSFECTION FOR GENERATING RECOMBINANT VIRUS procedure into primary chick embryo fibroblast (CEF) cells. A blue virus obtained from the transfection stock was purified by successive plaque purifications using the BLUOGAL SCREEN FOR RECOMBINANT HERPESVIRUS procedure. At the end of this procedure, when 100% of the plaques were blue, the DNA was analyzed for the presence of the *lacZ* gene. S-HVT-005 is a recombinant virus that contains the *lacZ* gene incorporated into the genome in place of the deleted gA gene of HVT.

TESTING OF RECOMBINANT S-HVT-005

S-HVT-005 may be formulated as a vaccine in the same manner as S-HVT-045. When administered to chickens, such a vaccine provides protection against Marek's disease virus.

Example 10

Marek's Disease Vaccines

Recombinant HVT expressing glycoproteins from Marek's Disease Virus make superior vaccines for Marek's Disease. We have constructed several recombinant HVT expressing MDV glycoproteins: S-HVT-004 (example 3), S-HVT-005 (example 4), S-HVT-045 (example 5), S-HVT-046 (example 10A), S-HVT-047 (example 10B), S-HVT-062 (example 10C).

Example 10A S-HVT-046

S-HVT-046 is a recombinant herpesvirus of turkeys that contains the Marek's disease virus (MDV) glycoprotein B (gB) and glycoprotein A (gA) genes inserted into the short unique region. The MDV genes are inserted in the same transcriptional orientation as the US2 gene. The MDV antigens are more likely to elicit the proper antigenic response than the HVT equivalent antigen.

S-HVT-046 was constructed according to the PROCEDURE FOR GENERATING RECOMBINANT HERPESVIRUS FROM SUBGENOMIC DNA FRAGMENTS. The following combination of subgenomic clones and enzymes were used: 407-32.2C3 with *Not*I, 172-07.BA2 with *Bam*HI, 407-32.5G6 with *Not*I, 407-32.1C1 with *Not*I, 437-26.24 with *Bam*HI and *Hind*III, 437-26.26 with *Bam*HI and *Hind*III, and 456-17.22 uncut. Insertion of the appropriate DNA was confirmed by southern blot analysis.

Example 10B S-HVT-047

S-HVT-047 is a recombinant herpesvirus of turkeys that

contains the MDV gB and gA genes inserted into the short unique region. The MDV genes are inserted in the opposite transcriptional orientation as the US2 gene. The MDV antigens are more likely to elicit the proper antigenic response than the HVT equivalent antigen.

S-HVT-047 was constructed according to the PROCEDURE FOR GENERATING RECOMBINANT HERPESVIRUS FROM SUBGENOMIC DNA FRAGMENTS. The following combination of subgenomic clones and enzymes were used: 407-32.2C3 with *Not*I, 172-07.BA2 with *Bam*HI, 407-32.5G6 with *Not*I, 407-32.1C1 with *Not*I, 437-26.24 with *Bam*HI and *Hind*III, 437-26.26 with *Bam*HI and *Hind*III, and 456-17.18 uncut. Insertion of the appropriate DNA was confirmed by southern blot analysis.

Example 10C S-HVT-062

S-HVT-062 is a recombinant herpesvirus of turkeys that contains the MDV gB, glycoprotein D (gD) and gA genes inserted into the short unique region. The MDV genes are inserted in the same transcriptional orientation as the US2 gene. The MDV antigens are more likely to elicit the proper antigenic response than the HVT equivalent antigen. S-HVT-062 has been deposited on February 23, 1993 pursuant to the Budapest Treaty on the International Deposit of Microorganisms for the Purposes of Patent Procedure with the Patent Culture Depository of the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852 U.S.A. under ATCC Accession No. VR 2401.

S-HVT-046 was constructed according to the PROCEDURE FOR GENERATING RECOMBINANT HERPESVIRUS FROM SUBGENOMIC DNA FRAGMENTS. The following combination of subgenomic clones and enzymes were used: 407-32.2C3 with *Not*I, 172-07.BA2 with *Bam*HI, 407-32.5G6 with *Not*I, 407-32.1C1 with *Not*I, 437-26.24

with *BamHI* and *HindIII*, 556-60.6 with *BamHI* and *HindIII*, and 456-17.22 uncut. Insertion of the appropriate DNA was confirmed by southern blot analysis.

TESTING OF RECOMBINANT HVT EXPRESSING MDV ANTIGENS

Two studies were conducted to demonstrate the effectiveness of these recombinant HVT/MDV viruses in protecting against challenge with virulent Marek's disease virus. In Study 1, one-day-old specific pathogen free (SPF) chicks were vaccinated with either S-HVT-045, S-HVT-046, or S-HVT-047. Five days post-vaccination, vaccinated chicks, and non-vaccinated, control chicks were challenged with MDV. Following a 6-week post-challenge observation period for clinical signs typical of Marek's disease, all chicks were necropsied and examined for lesions diagnostic of Marek's disease. The results, in Table 7, show these recombinant viruses gave complete protection against a challenge that caused Marek's disease in 84% of non-vaccinated control chicks.

In the second study, one-day-old chicks were vaccinated with S-HVT-062. Two more groups of chicks were vaccinated with a USDA-licensed, conventional vaccines comprised of HVT and a combination HVT and SB-1 viruses. Five days post-vaccination, the vaccinated chicks and a group of non-vaccinated, control chicks were challenged with MDV. The chicks were observed for 8 weeks for clinical signs of Marek's disease, then necropsied and observed for Marek's lesions. This study demonstrated the ability of S-HVT-062 to provide 100% protection against challenge (Table 7). The commercial vaccines gave 81% and 95% protection, respectively and 100% of the non-vaccinated chicks developed Marek's disease.

TABLE 7 EFFICACY OF RECOMBINANT HVT/MDV VIRUSES AGAINST VIRULENT MAREK'S VIRUS CHALLENGE

Study	Vaccine Group	Dose[a]	Protection[b]
1	S-HVT-045	2.2×10^3	24/24 (100%)
1	S-HVT-046	2.2×10^3	20/20 (100%)
1	S-HVT-047	2.2×10^3	24/24 (100%)
1	Controls		7/44 (16%)
1	HVT/SB-1		24/25 (96%)
2	S-HVT-062	7.5×10^2	32/32 (100%)
2	S-HVT-063	7.5×10^2	22/22 (100%)
2	Controls		0/20 (0%)
2	HVT[c]	7.5×10^2	17/21 (81%)
2	HVT/SB-1[c]	7.5×10^2	21/22 (95%)

[a] PFU/0.2 ml.

[b] No. protected/Total; Challenge 5 days post-vaccination.

[c] Commercial vaccine.

Example 11

Bivalent Vaccines Against Newcastle Disease and Marek's Disease

Recombinant HVT expressing proteins from NDV make bivalent vaccines protecting against both Marek's Disease and Newcastle disease. We have constructed several recombinant HVT expressing NDV proteins S-HVT-007 (example 11A), S-HVT-040 (example 11B), S-HVT-049 (example 11C), S-HVT-055 (example 11D),and S-HVT-106 (example 11E).

Example 11A S-HVT-007

S-HVT-007 is a recombinant herpesvirus of turkeys that contains a hybrid *E. coli* lacZ NDV HN hybrid protein gene under the control of the PRV gX promoter and the NDV F gene under the control of the HSV-1 a 4 promoter inserted into the long unique region. The NDV genes are inserted in the same transcriptional orientation as the UL43 gene.

To construct S-HVT-007 HVT DNA and the plasmid 255-18.B16 were co-transfected according to the DNA TRANSFECTION FOR GENERATING RECOMBINANT VIRUS procedure into primary chick embryo fibroblast (CEF) cells. A blue virus obtained from the transfection stock was purified by successive plaque purifications using the BLUOGAL SCREEN FOR RECOMBINANT HERPESVIRUS procedure. At the end of this procedure, when 100% of the plaques were blue.

Example 11B S-HVT-048

S-HVT-048 is a recombinant herpesvirus of turkeys that contains the MDV gB and gA genes and the NDV F gene under the control of the HCMV immediate early promoter inserted into the short unique region. The MDV and NDV genes are inserted in the same transcriptional orientation as the US2 gene.

S-HVT-048 was constructed according to the PROCEDURE FOR GENERATING RECOMBINANT HERPESVIRUS FROM SUBGENOMIC DNA FRAGMENTS. The following combination of subgenomic clones and enzymes were used: 407-32.2C3 with *Not*I, 172-07.BA2 with *Bam*HI, 407-32.5G6 with *Not*I, 407-32.1C1 with *Not*I, 437-26.24 with *Bam*HI and *Hind*III, 437-26.26 with *Bam*HI and *Hind*III, and 535-70.3 uncut. Insertion of the appropriate DNA was confirmed by southern blot analysis.

Example 11C S-HVT-049

S-HVT-049 is a recombinant herpesvirus of turkeys that contains the MDV gB and gA genes and the NDV HN gene under the control of the PRV gX promoter inserted into the short unique region. The MDV and NDV genes are inserted in the same transcriptional orientation as the US2 gene.

S-HVT-049 was constructed according to the PROCEDURE FOR GENERATING RECOMBINANT HERPESVIRUS FROM SUBGENOMIC DNA FRAGMENTS. The following combination of subgenomic clones and enzymes were used: 407-32.2C3 with *Not*I, 172-07.BA2 with *Bam*HI, 407-32.5G6 with *Not*I, 407-32.1C1 with *Not*I, 437-26.24 with *Bam*HI and *Hind*III, 437-26.26 with *Bam*HI and *Hind*III, and 549-62.10 uncut. Insertion of the appropriate DNA was confirmed by southern blot analysis.

Example 11D S-HVT-050

S-HVT-050 is a recombinant herpesvirus of turkeys that contains the MDV gB and gA genes and the NDV HN and F genes. The NDV genes are under the control of the PRV gX and HCMV immediately promoters respectively. All four genes are inserted into the short unique region in the same transcriptional orientation as the US2 gene.

S-HVT-050 was constructed according to the PROCEDURE FOR GENERATING RECOMBINANT HERPESVIRUS FROM SUBGENOMIC DNA FRAGMENTS. The following combination of subgenomic clones and enzymes were used: 407-32.2C3 with *Not*I, 172-07.BA2 with *Bam*HI, 407-32.5G6 with *Not*I, 407-32.1C1 with *Not*I, 437-26.24 with *Bam*HI and *Hin*dIII, 437-26.26 with *Bam*HI and *Hin*dIII, and 549-24.15 uncut. Insertion of the appropriate DNA was confirmed by southern blot analysis. S-HVT-050 has been deposited on February 23, 1993 pursuant to the Budapest Treaty on the International Deposit of Microorganisms for the Purposes of Patent Procedure with the Patent Culture Depository of the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852 U.S.A. under ATCC Accession No. VR 2400.

Example 11E S-HVT-106

S-HVT-106 is a recombinant herpesvirus of turkeys that contains the MDV gA, gB, gD genes and the NDV HN and F genes. The NDV genes are under the control of the PRV gX and HCMV immediately promoters respectively. All five genes are inserted into the short unique region in the same transcriptional orientation as the US2 gene.

S-HVT-050 was constructed according to the PROCEDURE FOR GENERATING RECOMBINANT HERPESVIRUS FROM SUBGENOMIC DNA FRAGMENTS. The following combination of subgenomic clones and enzymes were used: 407-32.2C3 with *Not*I, 172-07.BA2 with *Bam*HI, 407-32.5G6 with *Not*I, 407-32.1C1 with *Not*I, 437-26.24 with *Bam*HI and *Hin*dIII, 437-26.26 with *Bam*HI and *Hin*dIII, and 633-13.27 uncut.

TESTING OF RECOMBINANT HVT EXPRESSING NDV ANTIGENS

Two studies were conducted to demonstrate the effectiveness of these recombinant HVT/MDV/NDV viruses in protecting against challenge with virulent Newcastle and Marek's disease viruses. In Study 1, one-day-old specific pathogen free (SPF) chicks were vaccinated with either S-HVT-048, S-HVT-049, S-HVT-050, or a USDA-licensed, conventional vaccine comprised of NDV B1/B1 virus. Three weeks post-vaccination, vaccinated chicks, and non-vaccinated, control chicks were challenged with NDV. Birds were then observed for clinical signs of disease. The results, in Table 8, show these recombinant viruses (S-HVT-048 and S-HVT-050) gave complete against a challenge that caused Newcastle disease in 100% of non-vaccinated control chicks. Recombinant virus S-HVT-049 gave partial protection against Newcastle disease.

In the second study, one-day-old chicks were vaccinated with S-HVT-050. Two more groups of chicks were vaccinated with a USDA-licensed, conventional vaccines comprised of HVT and a combination HVT and SB-1 viruses. Five days post-vaccination, the vaccinated chicks and a group of non-vaccinated, control chicks were challenged with MDV. The chicks were observed for 8 weeks for clinical signs of Marek's disease, then necropsied and observed for Marek's lesions. This study demonstrated the ability of S-HVT-050 to provide protection greater than the commercial Marek's disease vaccines.

TABLE 8 EFFICACY OF RECOMBINANT HVT/MDV/NDV VIRUSES AGAINST VIRULENT NEWCASTLE AND MAREK'S DISEASE VIRUS CHALLENGE

Study	Vaccine Group	Dose[a]	Protection (%) NDV[b]	MDV[c]
1	S-HVT-048	4.0×10^4	19/19 (100)	
1	S-HVT-049	3.0×10^4	4/20 (20)	
1	S-HVT-050	1.5×10^4	20/20 (100)	
1	Controls		0/20 (0)	
1	NDV B1/B1[d]		18/18 (100)	
2	S-HVT-050	7.5×10^2		13/14 (93)
2	S-HVT-050	1.5×10^3		16/17 (94)
2	Controls			5/23 (22)
2	HVT[d]			20/26 (77)
2	HVT/SB-1[d]			10/12 (83)

a PFU/0.2 ml.

b No. protected/Total; Challenge 3 weeks post-vaccination.

c No. protected/Total; Challenge 5 days post-vaccination.

d Commercial vaccine.

Example 12

Bivalent Vaccines Against Infectious Laryngotracheitis and Marek's Disease

Recombinant HVT expressing glycoproteins from ILT virus make bivalent vaccines protecting against both Marek's disease and infectious laryngotracheitis. We have constructed several recombinant HVT expressing ILT virus glycoproteins S-HVT-051 (example 12A), S-HVT-052 (example 12B), and S-HVT-104 (example 11C).

Example 12A S-HVT-051

S-HVT-051 is a recombinant herpesvirus of turkeys that contains the ILT virus gB gene inserted into the short unique region. The ILT gene is inserted in the same transcriptional orientation as the US2 gene.

S-HVT-051 was constructed according to the PROCEDURE FOR GENERATING RECOMBINANT HERPESVIRUS FROM SUBGENOMIC DNA FRAGMENTS. The following combination of subgenomic clones and enzymes were used: 407-32.2C3 with *Not*I, 172-07.BA2 with *Bam*HI, 407-32.5G6 with *Not*I, 407-32.1C1 with *Not*I, 437-26.24 with *Bam*HI and *Hind*III, 437-26.26 with *Bam*HI and *Hind*III, and 528-11.34 uncut. Insertion of the appropriate DNA was confirmed by southern blot analysis.

Example 12B S-HVT-052

S-HVT-052 is a recombinant herpesvirus of turkeys that contains the ILT virus gD gene inserted into the short

unique region. The ILT gene is inserted in the opposite transcriptional orientation as the US2 gene.

S-HVT-052 was constructed according to the PROCEDURE FOR GENERATING RECOMBINANT HERPESVIRUS FROM SUBGENOMIC DNA FRAGMENTS. The following combination of subgenomic clones and enzymes were used: 407-32.2C3 with *Not*I, 172-07.BA2 with *Bam*HI, 407-32.5G6 with *Not*I, 407-32.1C1 with *Not*I, 437-26.24 with *Bam*HI and *Hind*III, 437-26.26 with *Bam*HI and *Hind*III, and 528-03.37 uncut. Insertion of the appropriate DNA was confirmed by southern blot analysis.

Example 12C S-HVT-104

S-HVT-104 is a recombinant herpesvirus of turkeys that contains six foreign genes. The MDV gA, gB, and gD genes are inserted in the unique short region in the same transcriptional orientation as the US2 gene. An *E. coli lacZ* marker gene and the ILT gB and gD genes are inserted in *Bam*HI #16 region in the same transcriptional orientation as the UL43 gene.

To construct S-HVT-104, DNA from S-HVT-062 and the plasmid 634-29.16 were co-transfected according to the DNA TRANSFECTION FOR GENERATING RECOMBINANT VIRUS procedure into primary chick embryo fibroblast (CEF) cells.

TESTING OF RECOMBINANT HVT EXPRESSING ILT ANTIGENS

The following study was conducted to demonstrate the effectiveness of these recombinant HVT/ILT viruses in protecting against challenge with virulent Infectious Laryngotracheitis virus. One-day-old specific pathogen

free (SPF) chicks were vaccinated with either S-HVT-051, S-HVT-052, a combination of S-HVT-051 and S-HVT-052, or a USDA-licensed, conventional vaccine comprised of ILT virus. Two to three weeks post-vaccination, vaccinated chicks, and non-vaccinated, control chicks were challenged with ILT. Birds were then observed for clinical signs of disease. The results, in Table 9, show these recombinant viruses (S-HVT-051 and S-HVT-052) gave protection against challenge with ILT virus comparable to a commercial ILT vaccine.

Animals vaccinated with the vaccines described here may be easily differentiated from animals infected with virulent ILT. This is accomplished by testing the suspect birds for antibodies to any ILT antigens other than gB or gD. Examples of such antigens are ILT glycoproteins C, E, and G. Vaccinated, uninfected birds will be negative for these antigens whereas infected birds will be positive.

TABLE 9 EFFICACY OF RECOMBINANT HVT/ILT VIRUSES AGAINST VIRULENT INFECTIOUS LARYNGOTRACHEITIS VIRUS CHALLENGE

Vaccine Group	Dose[a]	Protection[b]
S-HVT-051	2.1×10^3	28/30 (93%)
S-HVT-052	1.7×10^3	29/29 (100%)
S-HVT-051 + S-HVT-052	2.1×10^3 / 1.7×10^3	24/24 (100%)
Controls		2/30 (7%)
ILT[c]		29/30 (97%)

[a] PFU/0.2 ml.

[b] No.protected/Total; Challenge 2-3 weeks post-vaccination.

[c] Commercial vaccine.

Example 13

Bivalent Vaccines Against Infectious Bursal Disease and Marek's Disease

Recombinant HVT expressing proteins from IBDV make bivalent vaccines protecting against both Marek's Disease and infectious bursal disease. We have constructed several recombinant HVT expressing IBDV proteins. These viruses include S-HVT-003 (example 3) and S-HVT-096.

S-HVT-096 is a recombinant herpesvirus of turkeys that contains the IBDV VP2 gene, under the control of the HCMV immediate early promoter, inserted into the short unique region. The IBDV gene is inserted in the same transcriptional orientation as the US2 gene.

S-HVT-096 was constructed according to the PROCEDURE FOR GENERATING RECOMBINANT HERPESVIRUS FROM SUBGENOMIC DNA FRAGMENTS. The following combination of subgenomic clones and enzymes were used: 407-32.2C3 with *Not*I, 172-07.BA2 with *Bam*HI, 407-32.5G6 with *Not*I, 407-32.1C1 with *Not*I, 437-26.24 with *Bam*HI and *Hin*dIII, 556-60.6 with *Bam*HI, and 602-57.F1 uncut. Insertion of the appropriate DNA was confirmed by southern blot analysis.

TESTING OF RECOMBINANT HVT EXPRESSING IBDV ANTIGENS

S-HVT-096 was assayed for expression of VP2 by black plaque and western blot analysis. Both assays indicated that the virus was expressing high levels of protein which reacts specifically with an IBDV neutralizing monoclonal antibody. This virus will be useful as a vaccine against infectious bursal disease.

Example 14

Bivalent Vaccines Against Infectious Bronchitis and Marek's Disease

S-HVT-066 is a recombinant herpesvirus of turkeys that contains the MDV gB, gD and gA genes and the IBV spike and matrix genes. The IBV spike and matrix genes are under the control of the HCMV immediate early and PRV gX promoters respectively. All five genes are inserted into the short unique region. The MDV and IBV genes are inserted in the same transcriptional orientation as the US2 gene.

S-HVT-066 was constructed according to the PROCEDURE FOR GENERATING RECOMBINANT HERPESVIRUS FROM SUBGENOMIC DNA FRAGMENTS. The following combination of subgenomic clones and enzymes were used: 407-32.2C3 with *Not*I, 172-07.BA2 with *Bam*HI, 407-32.5G6 with *Not*I, 407-32.1C1 with *Not*I, 437-26.24 with *Bam*HI and *Hind*III, 556-60.6 with *Bam*HI, and 567-72.1D uncut. Insertion of the appropriate DNA was confirmed by southern blot analysis.

TESTING OF RECOMBINANT HVT EXPRESSING IBV ANTIGENS

S-HVT-066 was assayed for expression of the IBV spike protein by black plaque and western blot analysis. Both assays indicated that the virus was expressing high levels of protein which reacts specifically with an IBV neutralizing monoclonal antibody. This virus will be useful as a vaccine against infectious bronchitis.

Example 21

Vaccines utilizing HVT to express antigens from various pathogens.

We also anticipate that antigens from the following pathogens may also be utilized to develop poultry vaccines: Chick anemia agent, Avian encephalomyelitis virus, Avian reovirus, Avian paramyxoviruses, Avian influenza virus, Avian adenovirus, Fowl pox virus, Avian coronavirus, Avian rotavirus, Salmonella spp *E. coli*, Pasteurella spp, Haemophilus spp, Chlamydia spp, Mycoplasma spp, Campylobacter spp, Bordetella spp, Poultry nematodes, cestodes, trematodes, Poultry mites/lice, Poultry protozoa (Eimeria spp, Histomonas spp, Trichomonas spp).

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What is claimed is:

1. A recombinant herpesvirus of turkeys comprising a foreign gene inserted within the genomic DNA of herpesvirus of turkeys, wherein the foreign gene: (a) is inserted with the unique *StuI* site in the US2 gene coding region of the herpesvirus of turkeys genomic DNA, and (b) is capable of being expressed in a host cell infected with herpesvirus of turkeys.

2. The recombinant herpesvirus of turkeys of claim 1, wherein the foreign gene encodes a polypeptide.

3. The recombinant herpesvirus of turkeys of claim 2, wherein the polypeptide is antigenic in an animal into which the recombinant herpesvirus is introduced.

4. The recombinant herpesvirus of turkeys of claim 2, wherein the polypeptide is *E. coli* beta-galactosidase.

5. The recombinant herpesvirus of turkeys of claim 4, designated S-HVT-012.

6. The recombinant herpesvirus of turkeys of claim 3, wherein the foreign gene encoding antigenic polypeptide is from Marek's disease virus, Newcastle disease virus, infectious laryngotracheitis virus, infectious bronchitis virus or infectious bursal disease virus.

7. The recombinant herpesvirus of turkeys of claim 6, wherein the foreign gene encoding antigenic polypeptide is from Marek's disease virus and encodes Marek's disease virus glycoprotein gA,

Marek's disease virus glycoprotein gB or Marek's disease virus glycoprotein gD.

8. The recombinant herpesvirus of claim 7, wherein the antigenic polypeptide is Marek's disease virus glycoprotein gB.

9. The recombinant herpesvirus of turkeys of claim 8, designated S-HVT-045.

10. The recombinant herpesvirus of turkeys of claim 8, further comprising foreign gene which encodes Marek's disease virus glycoprotein gA.

11. The recombinant herpesvirus of turkeys of claim 10 designated S-HVT-046.

12. The recombinant herpesvirus of turkeys of claim 10 designated S-HVT-047.

13. The recombinant herpesvirus of turkeys of claim 10, further comprising foreign DNA which encodes Marek's disease virus glycoprotein gD.

14. The recombinant herpesvirus of turkeys of claim 13 designated S-HVT-062.

15. The recombinant herpesvirus of turkeys of claim 6, wherein the foreign gene is from Newcastle disease virus and encodes Newcastle disease virus fusion protein or Newcastle disease virus hemagglutinin-neuraminidase.

16. The recombinant herpesvirus of turkeys of claim 15, wherein the antigenic polypeptide is Newcastle disease virus fusion protein.

17. The recombinant herpesvirus of turkeys of claim 15, wherein the antigenic polypeptide is Newcastle disease virus hemagglutinin-neuraminidase.

18. The recombinant herpesvirus of turkeys of claim 15, further comprising a foreign gene from Marek's disease virus, wherein the foreign gene encodes Marek's disease virus glycoprotein gA, Marek's disease virus glycoprotein gB or Marek's disease virus glycoprotein gD.

19. The recombinant herpesvirus of turkeys of claim 16, further comprising a foreign gene which encodes Marek's disease virus glycoprotein gA and a foreign gene which encodes Marek's disease virus glycoprotein gB, wherein both of the Marek's virus genes are inserted into the unique *StuI* site of the US2 gene coding region of the herpesvirus of turkeys genome.

20. The recombinant herpesvirus of turkeys of claim 19 designated S-HVT-048.

21. The recombinant herpesvirus of turkeys of claim 17, further comprising a foreign gene which encodes Marek's disease virus glycoprotein gB and a foreign gene which encodes Marek's disease virus glycoprotein gA, wherein both of the Marek's disease virus genes are inserted into the unique *StuI* site of the US2 gene coding region of the herpesvirus of turkeys genome.

22. The recombinant herpesvirus of turkeys of claim 21 designated S-HVT-049.

23. The recombinant herpesvirus of turkeys of claim 16, further comprising a foreign gene which encodes

Newcastle disease virus hemagglutinin-neuraminidase, wherein the hemagglutinin neuraminidase gene is inserted into the unique *StuI* site of the US2 gene coding region of the herpesvirus of turkeys genome.

24. The recombinant herpesvirus of turkeys of claim 23, further comprising a foreign gene which encodes Marek's disease virus glycoprotein gA and a foreign gene which encodes Marek's disease virus glycoprotein gB, wherein both of the Marek's disease virus genes are inserted into the unique *StuI* site of the US2 gene coding region of the herpesvirus of turkeys genome.

25. The recombinant herpesvirus of turkeys of claim 26 designated S-HVT-050.

26. The recombinant herpesvirus of claim 24, further comprising a foreign gene which encodes Marek's disease virus glycoprotein gD inserted into the unique *StuI* site of the US2 gene coding region of the herpesvirus of turkeys genome.

27. The recombinant herpesvirus of turkeys of claim 26 designated S-HVT-106.

28. The recombinant herpesvirus of turkeys of claim 6, wherein the foreign gene antigenic is from infectious laryngotracheitis virus and encodes infectious laryngotracheitis virus glycoprotein gB or infectious laryngotracheitis virus glycoprotein gD.

29. The recombinant herpesvirus of turkeys of claim 28, wherein the foreign gene encodes infectious laryngotracheitis virus glycoprotein gB.

30. The recombinant herpesvirus of turkeys of claim 29 designated S-HVT-051.

31. The recombinant herpesvirus of turkeys of claim 28, wherein the foreign gene encodes infectious laryngotracheitis virus glycoprotein gD.

32. The recombinant herpesvirus of turkeys of claim 31 designated S-HVT-052.

33. The recombinant herpesvirus of turkeys of claim 31, further comprising a foreign gene, which encodes infectious laryngotracheitis virus glycoprotein gB, inserted into the unique *Stu*I site of the US2 gene coding region of the herpesvirus of turkeys genome.

34. The recombinant herpesvirus of turkeys of claim 28, further comprising a foreign gene from Marek's disease virus, wherein the foreign gene encodes Marek's disease virus glycoprotein gA, Marek's disease virus glycoprotein gB or Marek's disease virus glycoprotein gD.

35. The recombinant herpesvirus of turkeys of claim 6, wherein the foreign gene is from infectious bronchitis virus and encodes infectious bronchitis virus spike protein or infectious bronchitis virus matrix protein.

36. The recombinant herpesvirus of turkeys of claim 35, wherein the foreign gene encodes infectious bronchitis virus spike protein.

37. The recombinant herpesvirus of turkeys of claim 36, further comprising a foreign gene encoding infectious bronchitis virus matrix protein, inserted

into the unique StuI site of the US2 gene coding region of the herpesvirus of turkeys genome.

38. The recombinant herpesvirus of turkeys of claim 37, further comprising a foreign gene from Marek's disease virus, wherein the foreign gene encodes Marek's disease virus glycoprotein gA, Marek's disease virus glycoprotein gB or Marek's disease virus glycoprotein gD.

39. The recombinant herpesvirus of turkeys of claim 37, further comprising a foreign gene which encodes Marek's disease virus glycoprotein gB, Marek's disease virus glycoprotein gA and Marek's disease virus glycoprotein gD, wherein the Marek's disease virus genes are inserted into the unique *StuI* site of the US2 gene of the herpesvirus of turkeys genome.

40. The recombinant herpesvirus of turkeys of claim 39 designated S-HVT-066.

41. The recombinant herpesvirus of turkeys of claim 6, wherein the foreign gene is from infectious bursal disease virus.

42. The recombinant herpesvirus of turkeys of claim 41, wherein the foreign gene encodes infectious bursal disease virus VP2 gene.

43. The recombinant herpesvirus of turkeys of claim 42 designated S-HVT-096.

44. The recombinant herpesvirus of turkeys of claim 41, further comprising a foreign gene from Marek's disease virus, wherein the foreign gene encodes Marek's disease virus glycoprotein gA, Marek's

disease virus glycoprotein gB or Marek's disease virus glycoprotein gD.

45. The recombinant herpesvirus of turkeys of claim 1, wherein the foreign gene is under a control of a herpesvirus promoter.

46. The recombinant herpesvirus of turkeys of claim 45, wherein the promoter is selected from PRV gX, HSV-1 alpha 4, HCMV immediate early, MDV gA, MDV gB, MDV gD, ILT gB and ILT gD.

47. A homology vector for producing a recombinant herpesvirus of turkeys by inserting foreign DNA into the genomic DNA of a herpesvirus of turkey which comprises a double-stranded DNA molecule consisting essentially of:

a) double stranded foreign DNA not usually present within the herpesvirus of turkeys genomic DNA

b) at one end the foreign DNA, double-stranded herpesvirus of turkeys DNA homologous to genomic DNA located at one side of the unique StuI site of the US2 coding region of the herpesvirus of turkeys genomic DNA and

c) at the other end of the foreign DNA, double-stranded herpesvirus of turkeys DNA homologous to genomic DNA located at the other side of the unique *StuI* site of the US2 gene coding region of the herpesvirus of turkeys genomic DNA.

48. A homology vector of claim 47, wherein the polypeptide is antigenic in the animal into which the recombinant herpesvirus of turkeys is introduced.

49. A homology vector of claim 48, wherein the antigenic polypeptide is from infectious bursal disease virus, Marek's disease virus, Newcastle disease virus, infectious laryngotracheitis virus, or infectious bronchitis virus.

50. A homology vector of claim 49, wherein the antigenic polypeptide is infectious bursal disease virus polyprotein, infectious bursal disease virus VP2 protein, Marek's disease virus glycoprotein gB, Marek's disease virus glycoprotein gA, Marek's disease virus glycoprotein gD, Newcastle disease virus fusion protein, Newcastle disease virus hemagglutinin-neuraminidase, infectious laryngotracheitis virus glycoprotein gB, infectious laryngotracheitis virus glycoprotein gD, infectious bronchitis virus spike protein, or infectious bronchitis virus matrix protein.

51. A homology vector of claim 47, wherein the detectable marker is *E. coli* beta-galactosidase.

52. The homology vector of claim 45 designated 435-47.1.

53. A vaccine useful for immunizing a bird against Marek's disease virus which comprises an effective immunizing amount of the recombinant herpesvirus of turkeys of claim 7, 9, 10, 11, 12, 13, or 14 and a suitable carrier.

54. A multivalent vaccine useful for immunizing a bird against Marek's disease virus and Newcastle disease virus which comprises an effective immunizing amount of the recombinant herpesvirus of turkeys of claim 5, 15, 18, 19, 20, 21, 22, 23, 24, 25, 26 or 27.

55. A multivalent vaccine useful for immunizing a bird against Marek's disease virus and infectious laryngotracheitis virus which comprises an effective immunizing amount of the recombinant herpesvirus of turkeys of claim 28, 30, 32, 33, or 34 and a suitable carrier.

56. A multivalent vaccine useful for immunizing a bird against Marek's disease virus and infectious bronchitis virus which comprises an effective immunizing amount of the recombinant herpesvirus of turkeys of claim 35, 37, 38, 39 or 40.

57. A multivalent vaccine useful for immunizing a bird against Marek's disease virus and infectious bursal disease virus which comprises an effective immunizing amount of the recombinant herpesvirus of turkeys of claim 42, 43, or 44 and a suitable carrier.

58. A method of immunizing a bird against Marek's disease virus which comprises administering to the bird an effecting immunizing dose of the vaccine of claim 53.

59. A method of immunizing a bird against Marek's disease virus and Newcastle disease virus which comprises administering to the bird an effective immunizing dose of the vaccine of claim 54.

60. A method of immunizing a bird against Marek's disease virus and infectious laryngotracheitis virus which comprises administering to the bird an effective immunizing dose of the vaccine of claim 55.

61. A method of immunizing a bird against Marek's disease virus and infectious bronchitis virus which comprises administering to the bird an effective immunizing dose of the vaccine of claim 56.

62. A method of immunizing a bird against Marek's disease virus and infectious bursal disease virus which comprises administering to the bird an effecting immunizing dose of the vaccine of claim 57.

63. A host cell infected with the recombinant herpesvirus of turkey of claim 1.

64. A host cell of claim 63, wherein the host cell is an avian cell.

FIGURE 1A

BamHI fragments

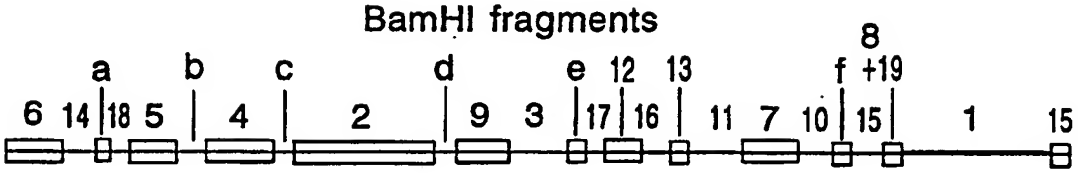


FIGURE 1B

BamHI #16

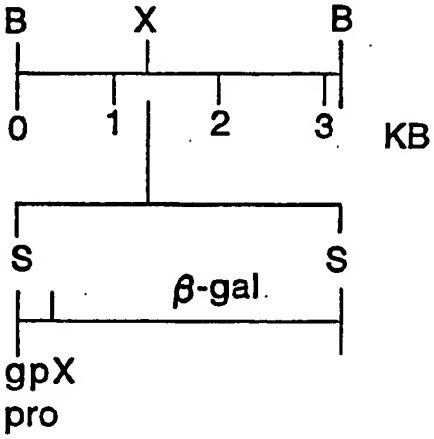


FIGURE 1C

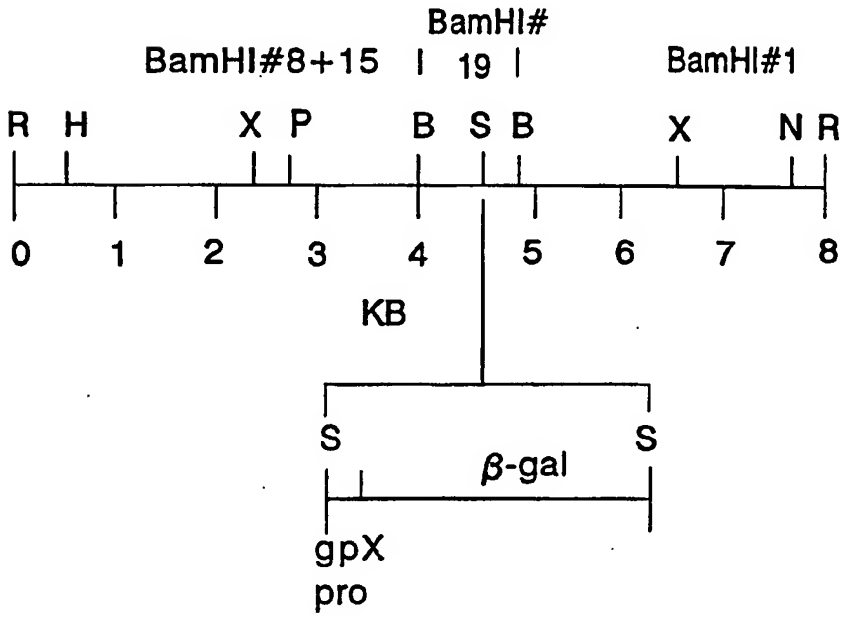


FIGURE 2A

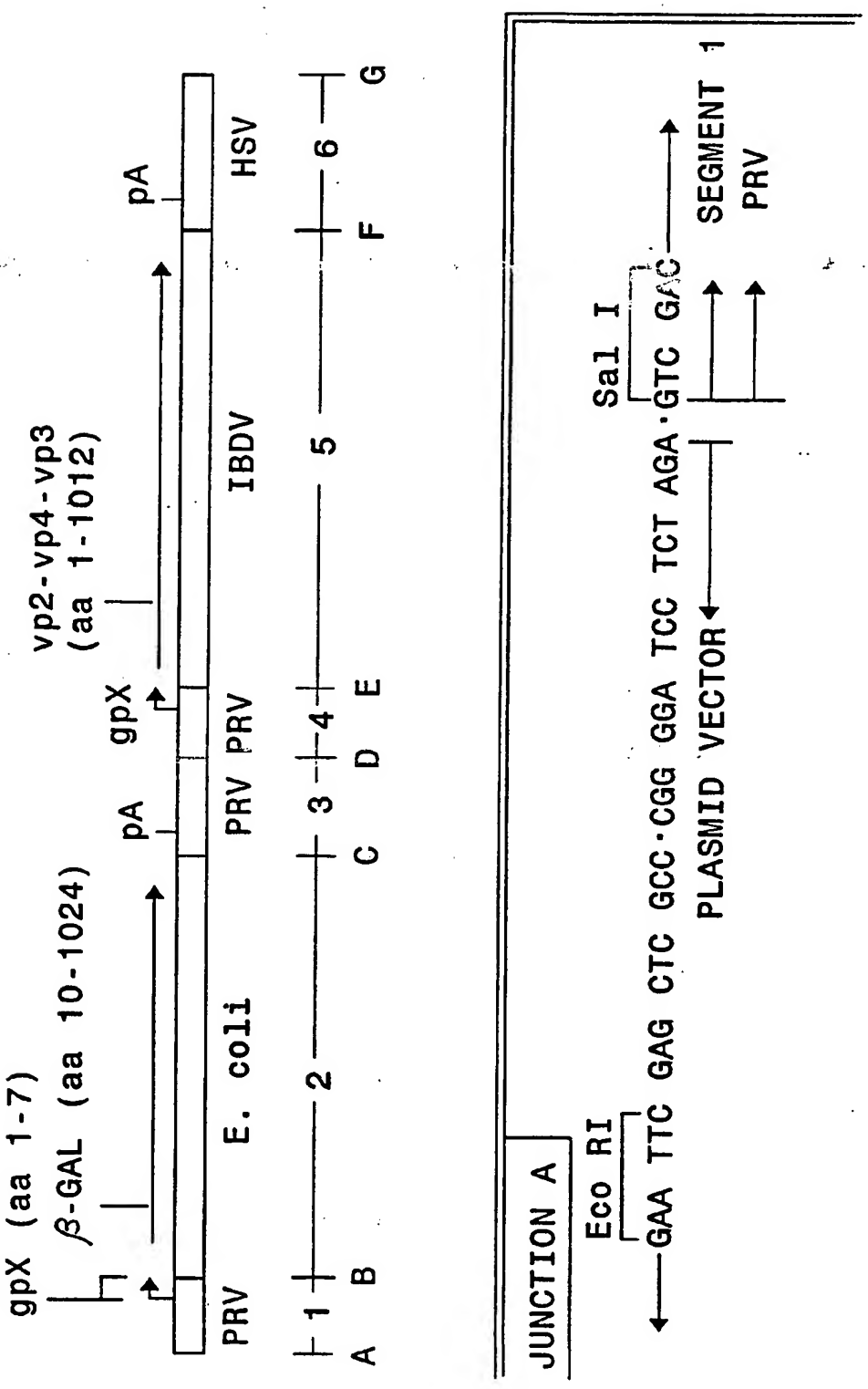
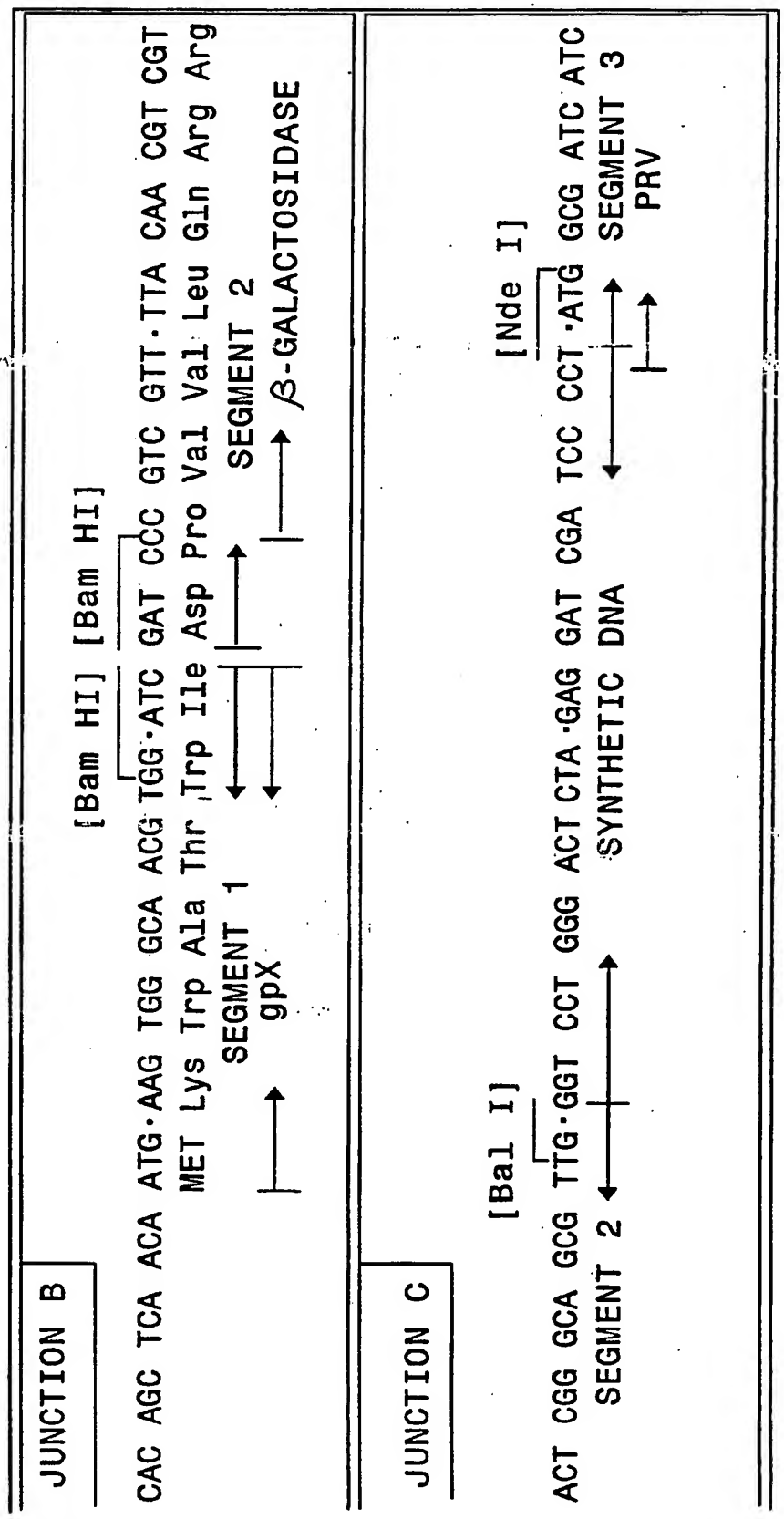

gpX (aa 1-7)
β-GAL (aa 10-1024)
pA
gpX
vp2-vp4-vp3
(aa 1-1012)
pA
PRV
E. coli
PRV PRV
IBDV
HSV
1
2
3
4
5
6
A B C D E F G
JUNCTION A
Eco RI
GAA TTC GAG CTC GCC·CGG GGA TCC TCT AGA·GTC GAC
Sal I
PLASMID VECTOR
SEGMENT 1
PRV

FIGURE 2B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MET Lys Trp Ala Thr Trp Ile Asp Pro Val Val Leu Gln Arg Arg
SEGMENT 1
gpX
SEGMENT 2
β-GALACTOSIDASE
JUNCTION C
[Bal I]
[Nde I]
ACT CGG GCA GCG TTG·GGT CCT GGG ACT CTA·GAG GAT CGA TCC CCT·ATG GCG ATC ATC
SEGMENT 2
SYNTHETIC DNA
SEGMENT 3
PRV

FIGURE 2C

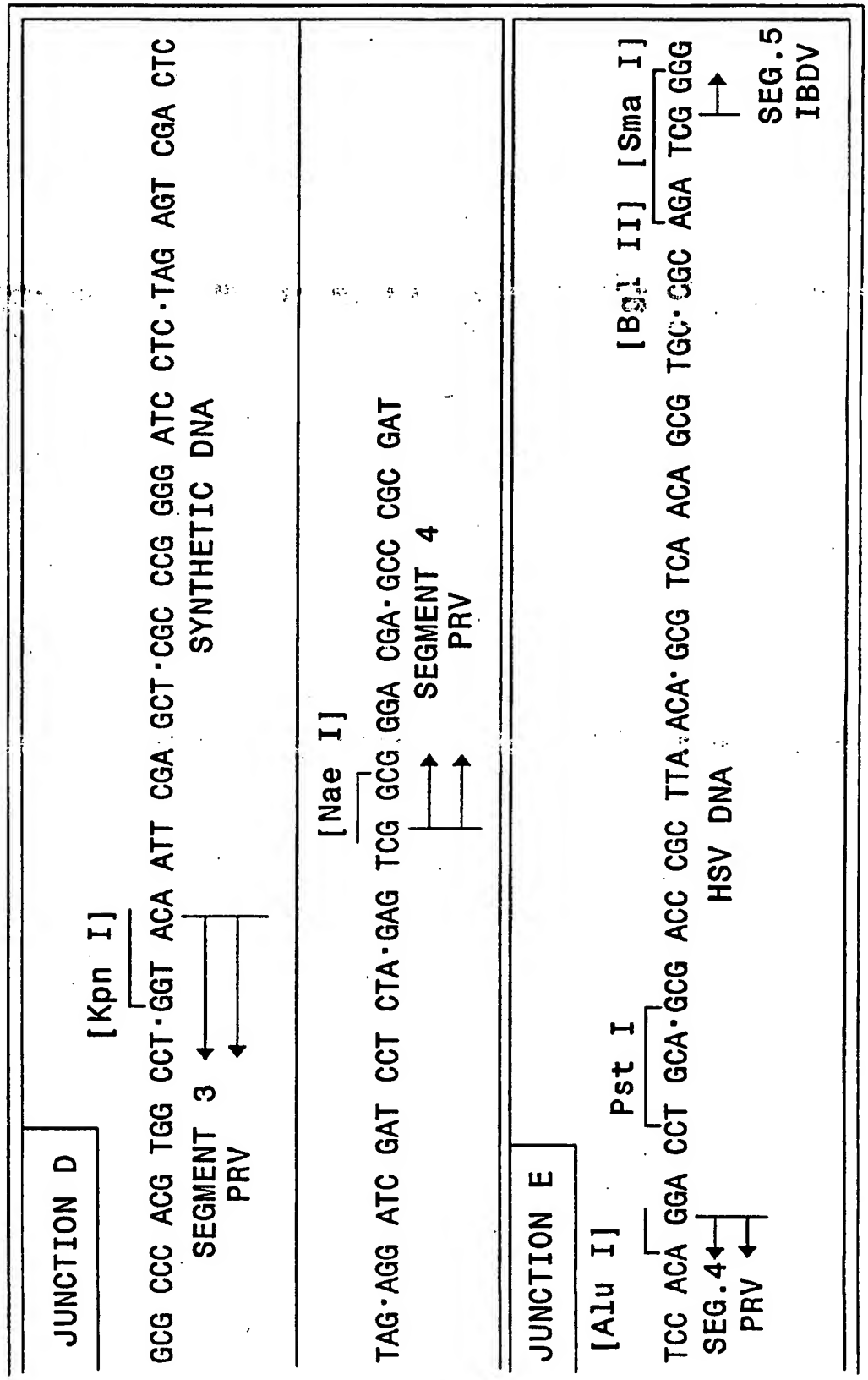
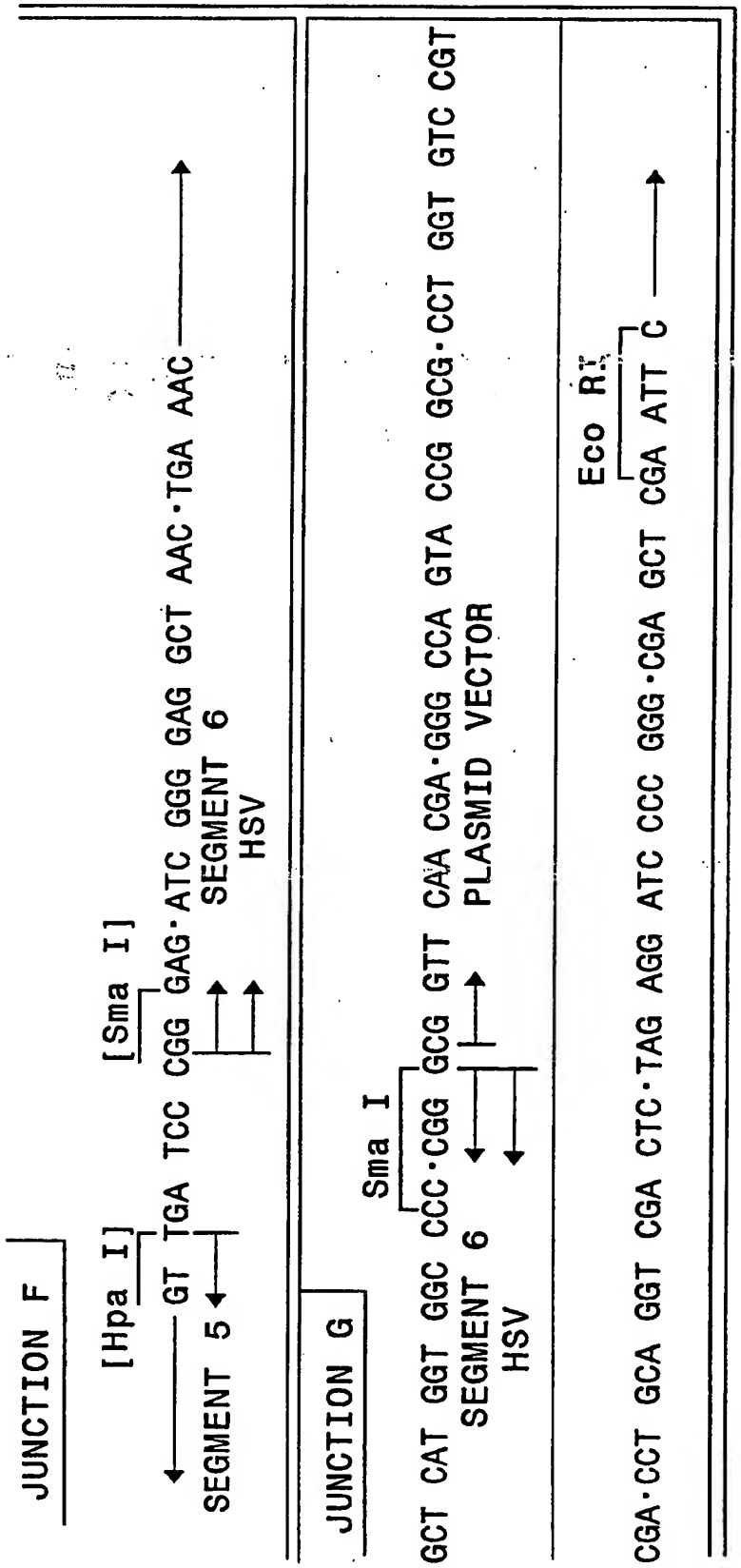

JUNCTION D
[Kpn I]
GCG CCC ACG TGG CCT·GGT ACA ATT CGA GCT·CGC CCG GGG ATC CTC·TAG AGT CGA CTC
SYNTHETIC DNA
SEGMENT 3
PRV
[Nae I]
TAG·AGG ATC GAT CCT CTA·GAG TCG GCG GGA CGA·GCC CGC GAT
SEGMENT 4
PRV
JUNCTION E
[Alu I]
Pst I
[Bgl II] [Sma I]
TCC ACA GGA CCT GCA·GCG ACC CGC TTA·ACA·GCG TCA ACA GCG TGC·GGC AGA TCG GGG
HSV DNA
SEG.4
PRV
SEG.5
IBDV

FIGURE 2D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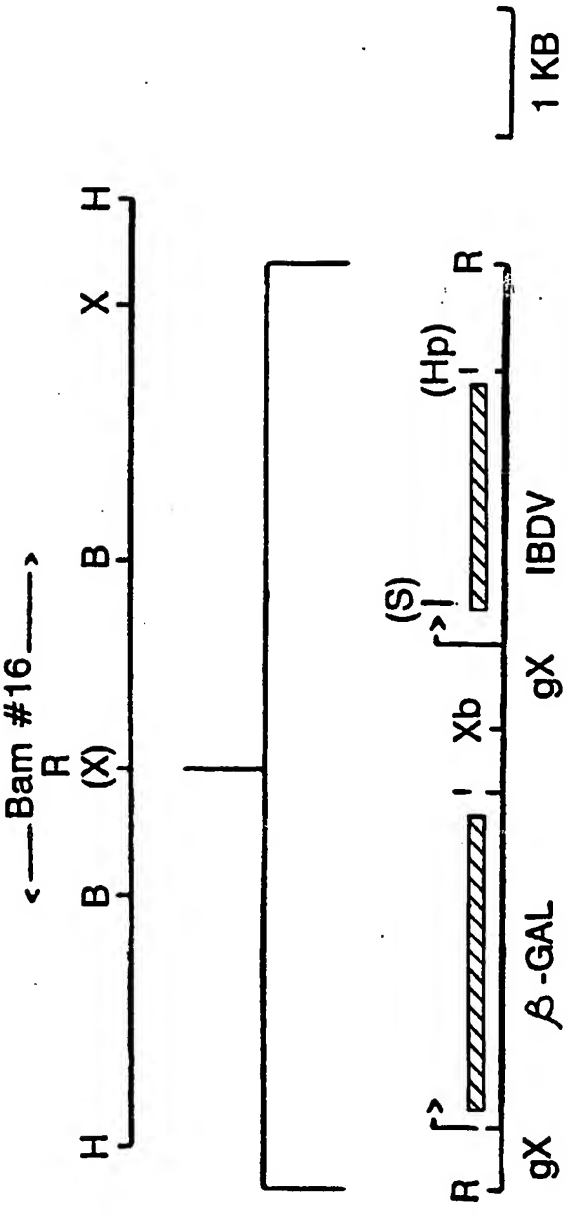
[Hpa I]
GT TGA TCC CGG GAG·ATC GGG GAG GCT AAC·TGA AAC
SEGMENT 5
[Sma I]
SEGMENT 6
HSV
JUNCTION G
Sma I
GCT CAT GGT GGC CCC·CGG GCG GTT CAA CGA·GGG CCA GTA CCG GCG·CCT GGT GTC CGT
SEGMENT 6
HSV
PLASMID VECTOR
Eco R I
CGA·CCT GCA GGT CGA CTC·TAG AGG ATC CCC GGG·CGA GCT CGA ATT C

FIGURE 3A



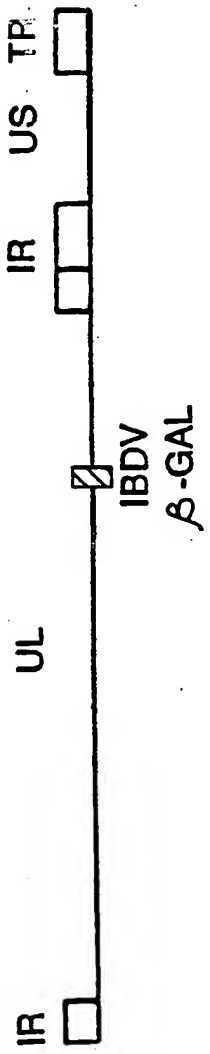


FIGURE 3B

FIGURE 4

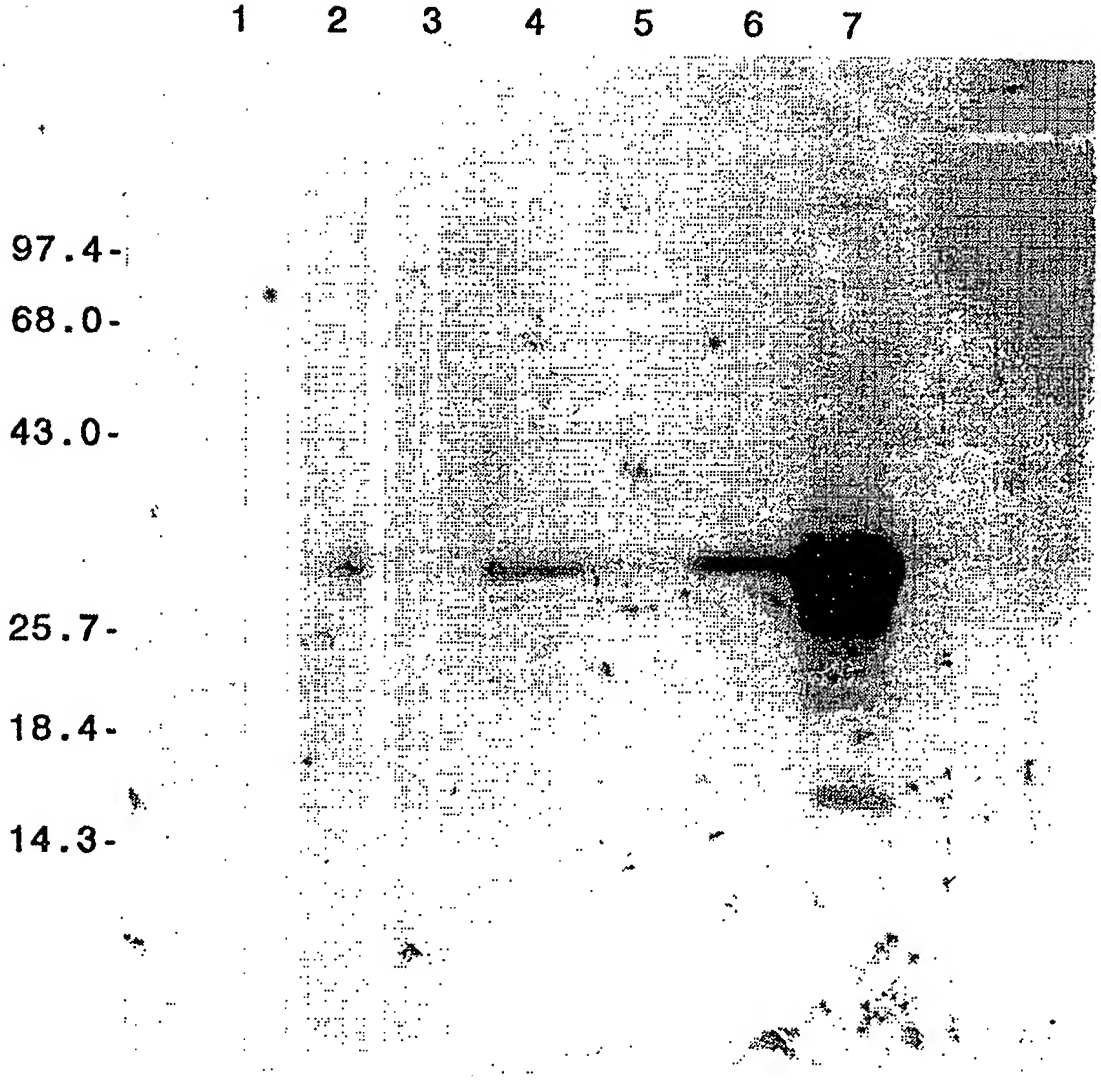
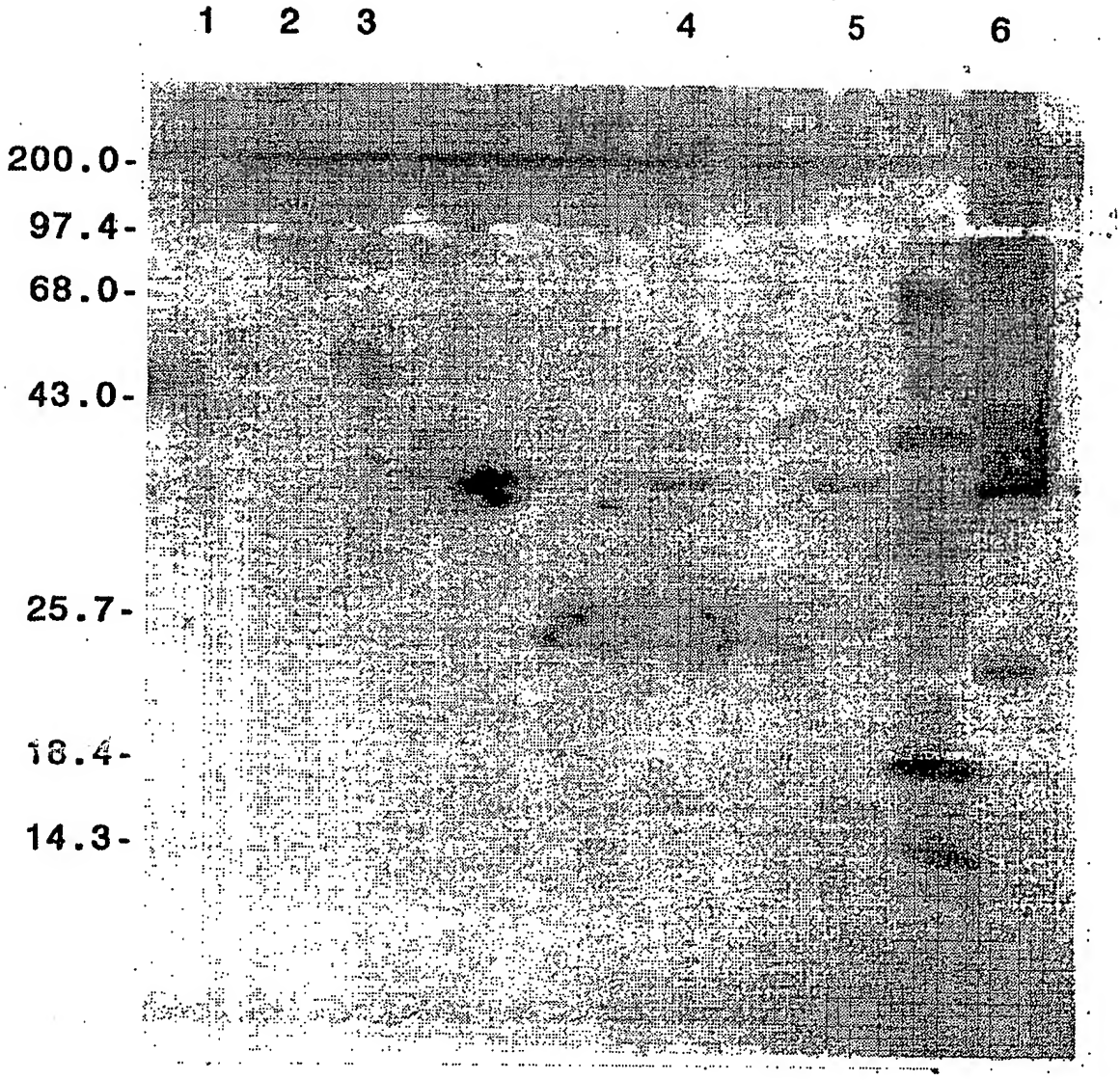


FIGURE 5



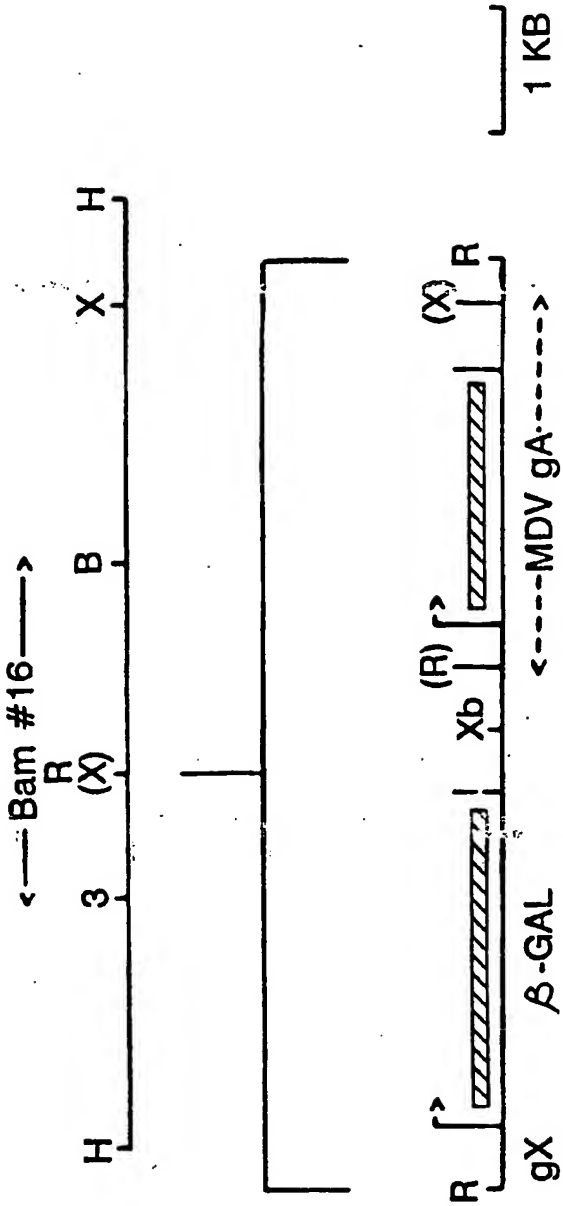


FIGURE 6A

FIGURE 6B

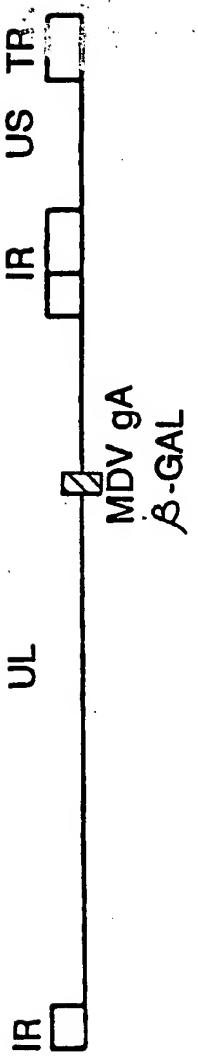
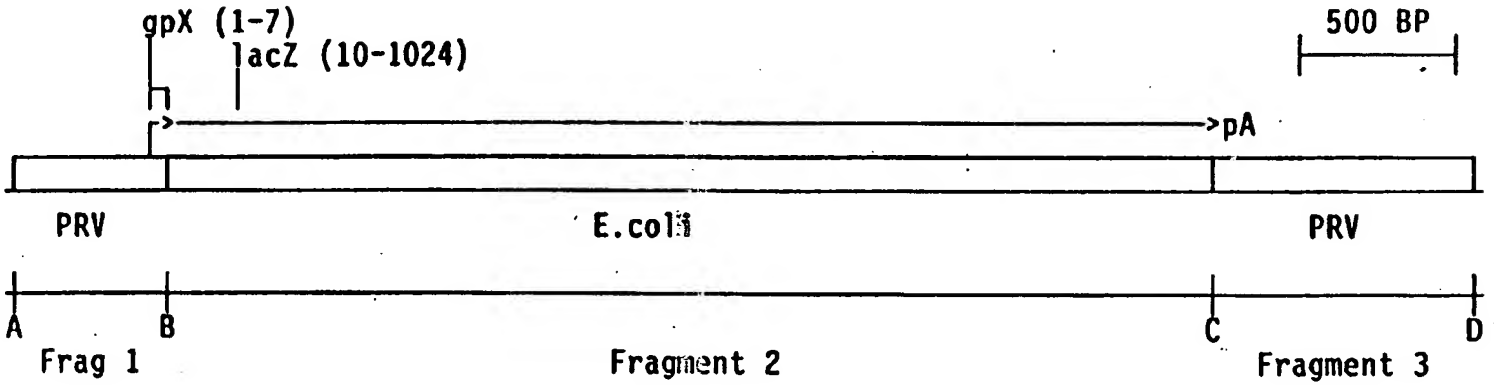
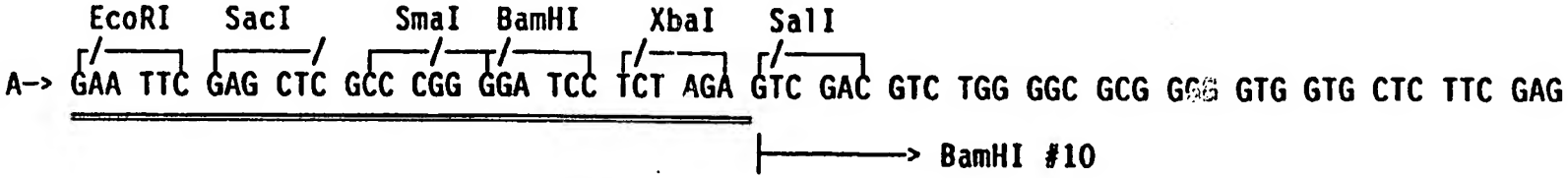


FIGURE 6C

FIGURE 7A


gpX (1-7)
lacZ (10-1024)
500 BP
pA
PRV
E.coli
PRV
A
B
C
D
Frag 1
Fragment 2
Fragment 3


EcoRI
SacI
SmaI
BamHI
XbaI
SalI
A-> GAA TTC GAG CTC GCC CGG GGA TCC TCT AGA GTC GAC GTC TGG GGC GCG GGG GTG GTG CTC TTC GAG
BamHI #10

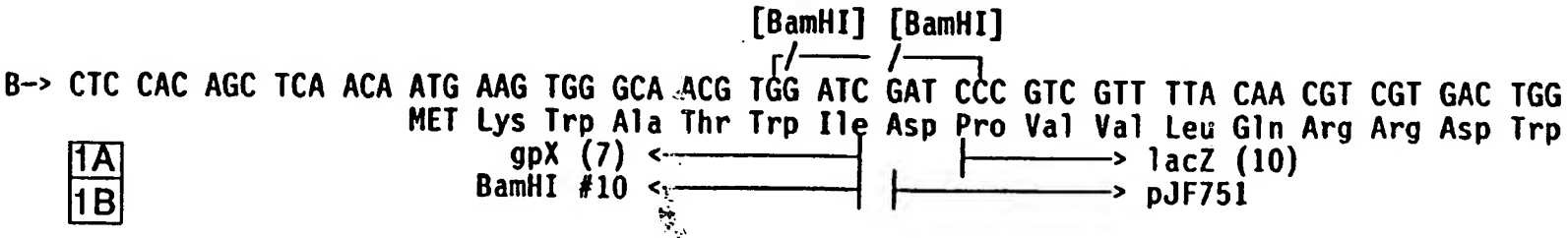

[BamHI] [BamHI]
B-> CTC CAC AGC TCA ACA ATG AAG TGG GCA ACG TGG ATC GAT CCC GTC GTT TTA CAA CGT CGT GAC TGG
MET Lys Trp Ala Thr Trp Ile Asp Pro Val Val Leu Gln Arg Arg Asp Trp
1A
1B
gpX (7)
BamHI #10
lacZ (10)
pJF751

FIGURE 7B

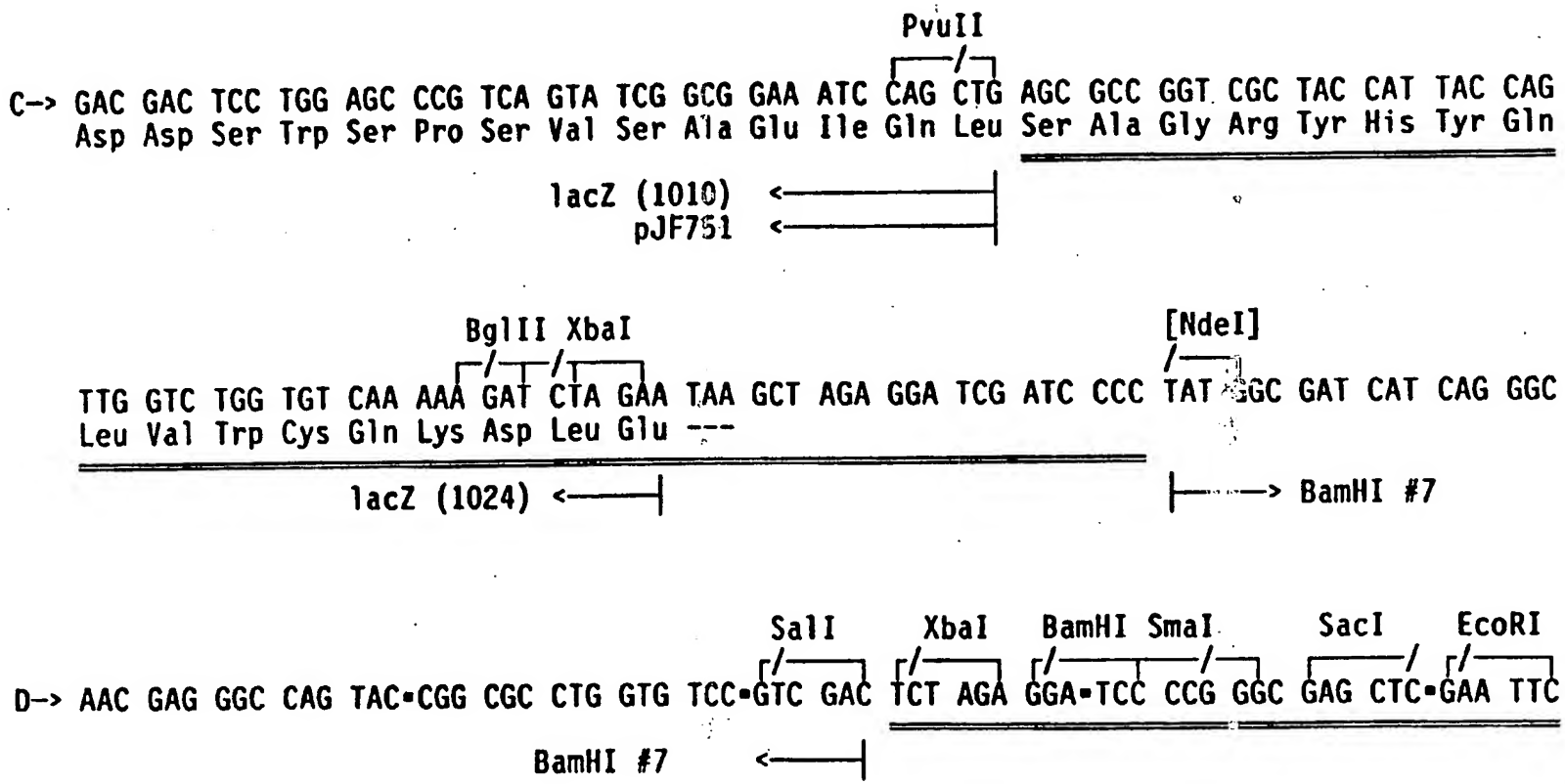

PvuII
C-> GAC GAC TCC TGG AGC CCG TCA GTA TCG GCG GAA ATC CAG CTG AGC GCC GGT CGC TAC CAT TAC CAG
Asp Asp Ser Trp Ser Pro Ser Val Ser Ala Glu Ile Gln Leu Ser Ala Gly Arg Tyr His Tyr Gln
lacZ (1010)
pJF751
BglII XbaI
[NdeI]
TTG GTC TGG TGT CAA AAA GAT CTA GAA TAA GCT AGA GGA TCG ATC CCC TAT GGC GAT CAT CAG GGC
Leu Val Trp Cys Gln Lys Asp Leu Glu ---
lacZ (1024)
BamHI #7
SalI
XbaI
BamHI SmaI
SacI
EcoRI
D-> AAC GAG GGC CAG TAC•CGG CGC CTG GTG TCC•GTC GAC TCT AGA GGA•TCC CCG GGC GAG CTC•GAA TTC
BamHI #7

FIGURE 8

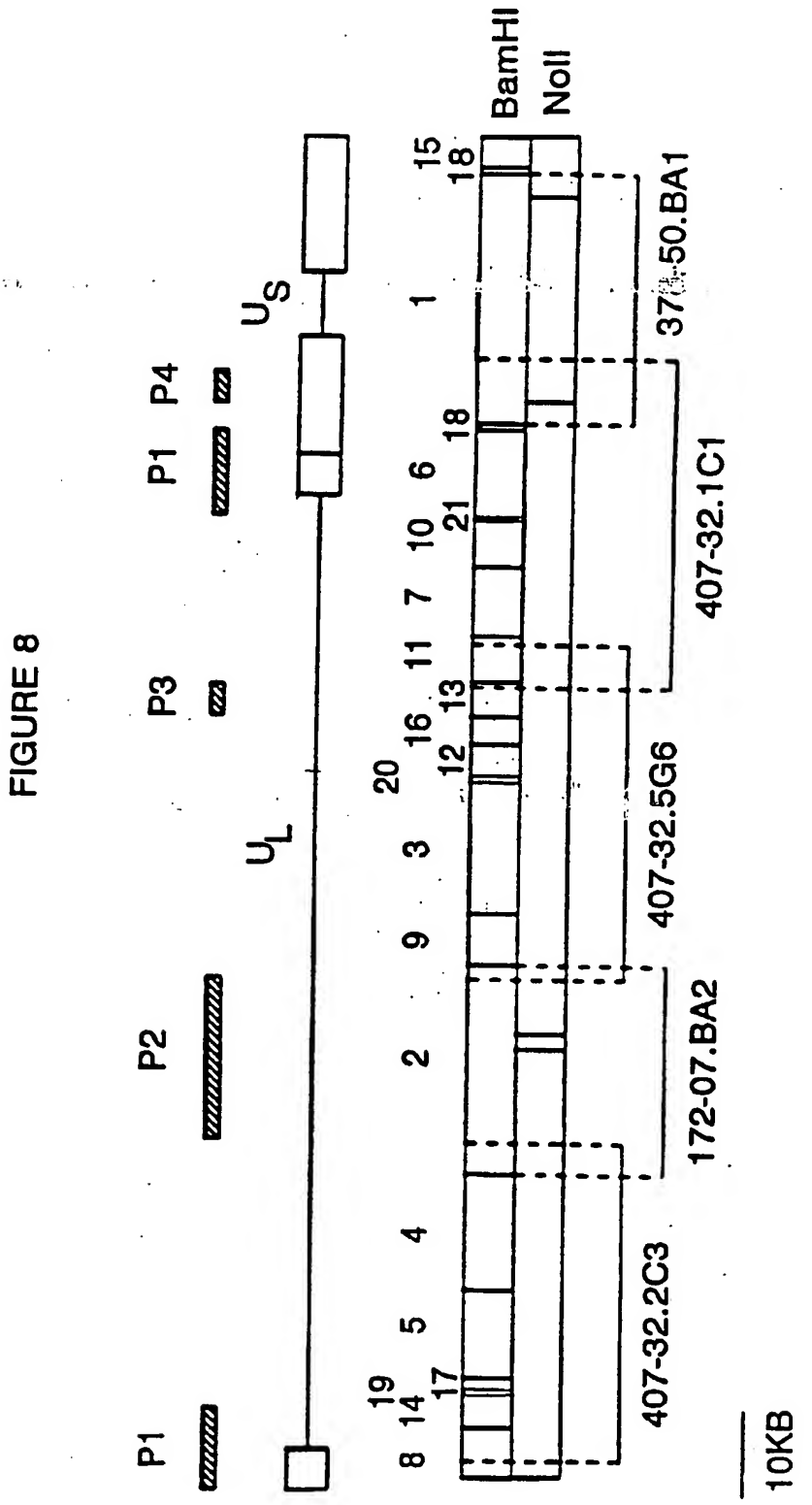

BamHI
NoII
P1
P2
P3
P1 P4
U_L
U_S
1 18 6 21 10 7 11 13 16 12 20 3 9 2 4 5 17 19 14 8 18 15
407-32.1C1
407-32.5G6
172-07.BA2
407-32.2C3
10KB

FIGURE 9

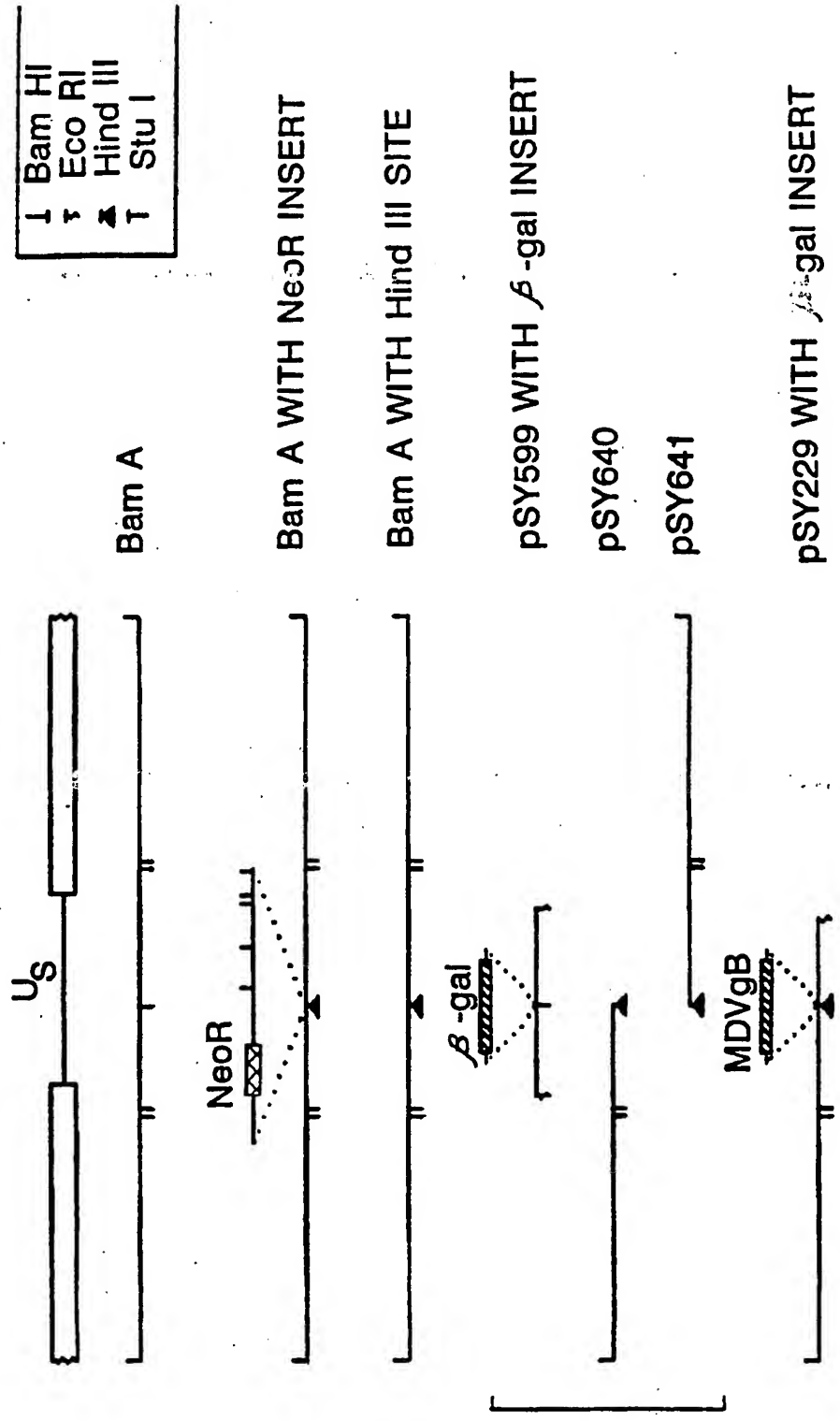
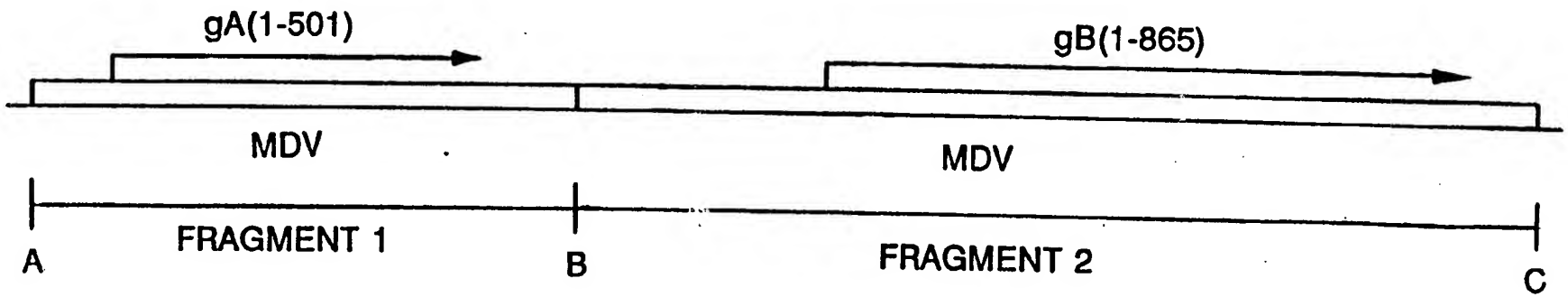

Bam HI
Eco RI
Hind III
Stu I
Bam A
Bam A WITH NeoR INSERT
Bam A WITH Hind III SITE
pSY599 WITH β-gal INSERT
pSY640
pSY641
pSY229 WITH β-gal INSERT
Us
NeoR
β-gal
MDVgB

FIGURE 10A



PsiI [PvuII]

JUNCTION A CAGGTCGAAGCTTGGGCGCTGCCTATGTAGTGAAATCTATACTGGGATTTATCATAACTAGTTTA

LINKER ◄ | | ► FRAGMENT 1
MDV

[EcoRV] [SalI]

JUNCTION B AATAATCTATCACTTTGTCATGGAGATGCCCAAGCTTCGACGACTCCCTTGGCCATGATGAATGG

FRAGMENT 1 ◄ | LINKER | ► FRAGMENT 2
MDV ◄ | ► MDV

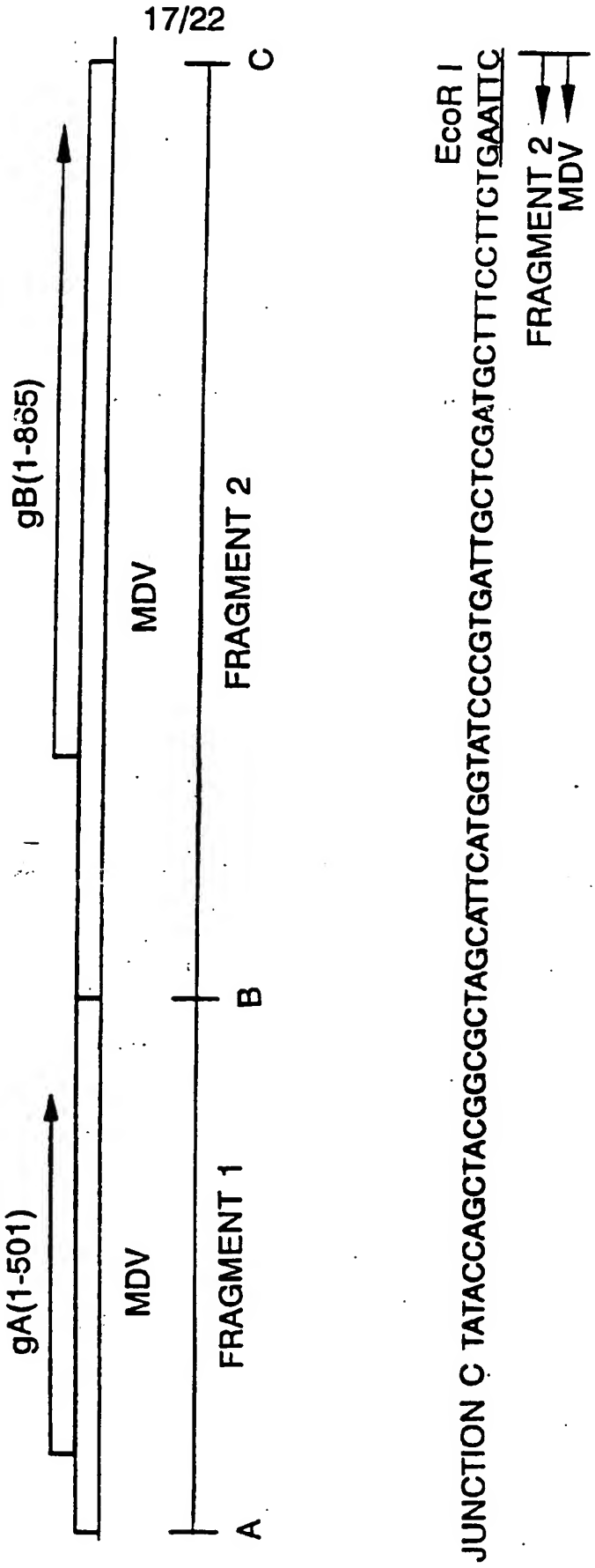
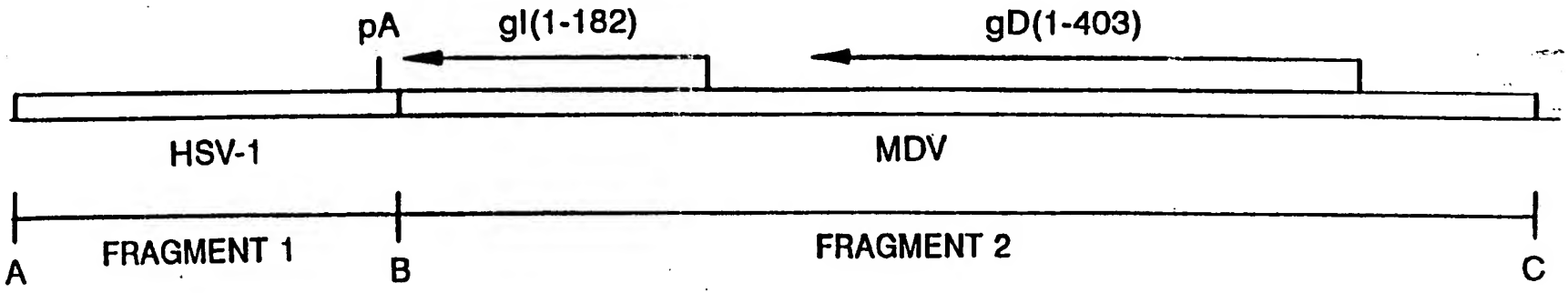

gA(1-501)
MDV
FRAGMENT 1
A
B
gB(1-865)
MDV
FRAGMENT 2
C
EcoR I
JUNCTION C TATACCAGCTACGGCGCTAGCATTCATGGTATCCCGTGATTGCTCGATGCTTTCCTTGTGAATTC
FRAGMENT 2
MDV

FIGURE 10B

FIGURE 11A



HindIII [SmaI]

JUNC.A AAGCTTGGCCTCGTCGTTAATTAACCCAATTCGAGCTCGCCCAGCTTGGGCTGCAGGTCGGGAAC

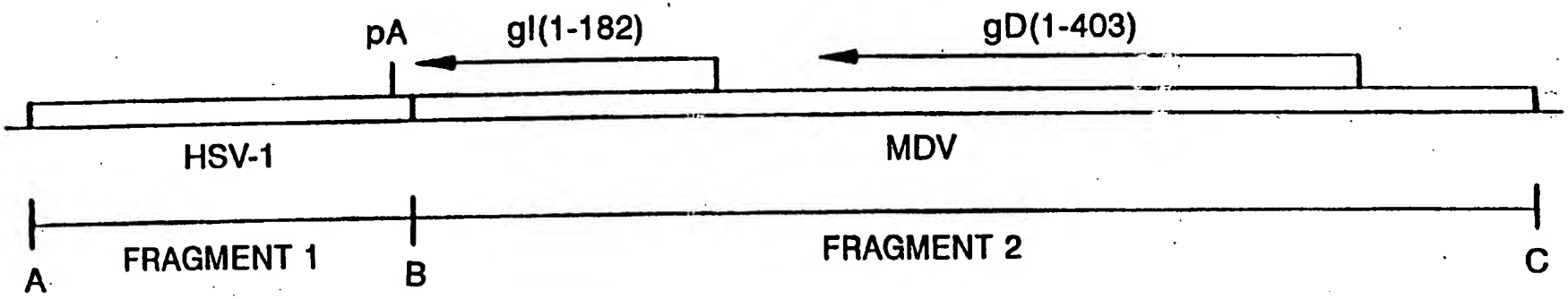
LINKER ◄ | ► FRAG. 1 HSV-1

[SmaI] [SalI]

JUNC.B TGTTTCAGTTAGCCTCCCCCATCTCCCGACTCTAGAGGATCTCGACATAGCGAATACATTTATGG

FRAGMENT 1 HSV-1 ◄ | LINKER | ► FRAGMENT 2 MDV

FIGURE 11B



Nco I

JUNC.C AACGTATATATTTTTCACGACGTAGACCACTATTGCCATGGACTCTAGAGGATCGGGTACCGAGC

FRAGMENT 2 MDV ← | | → LINKER

Hind III

JUNC.C CONT. TCGAATTGGGAAGCTTGTCGACTTAATTAAGCGGCCGCGTTTAAACGGCCCTCGAGGCCAAGCTT

FIGURE 12A

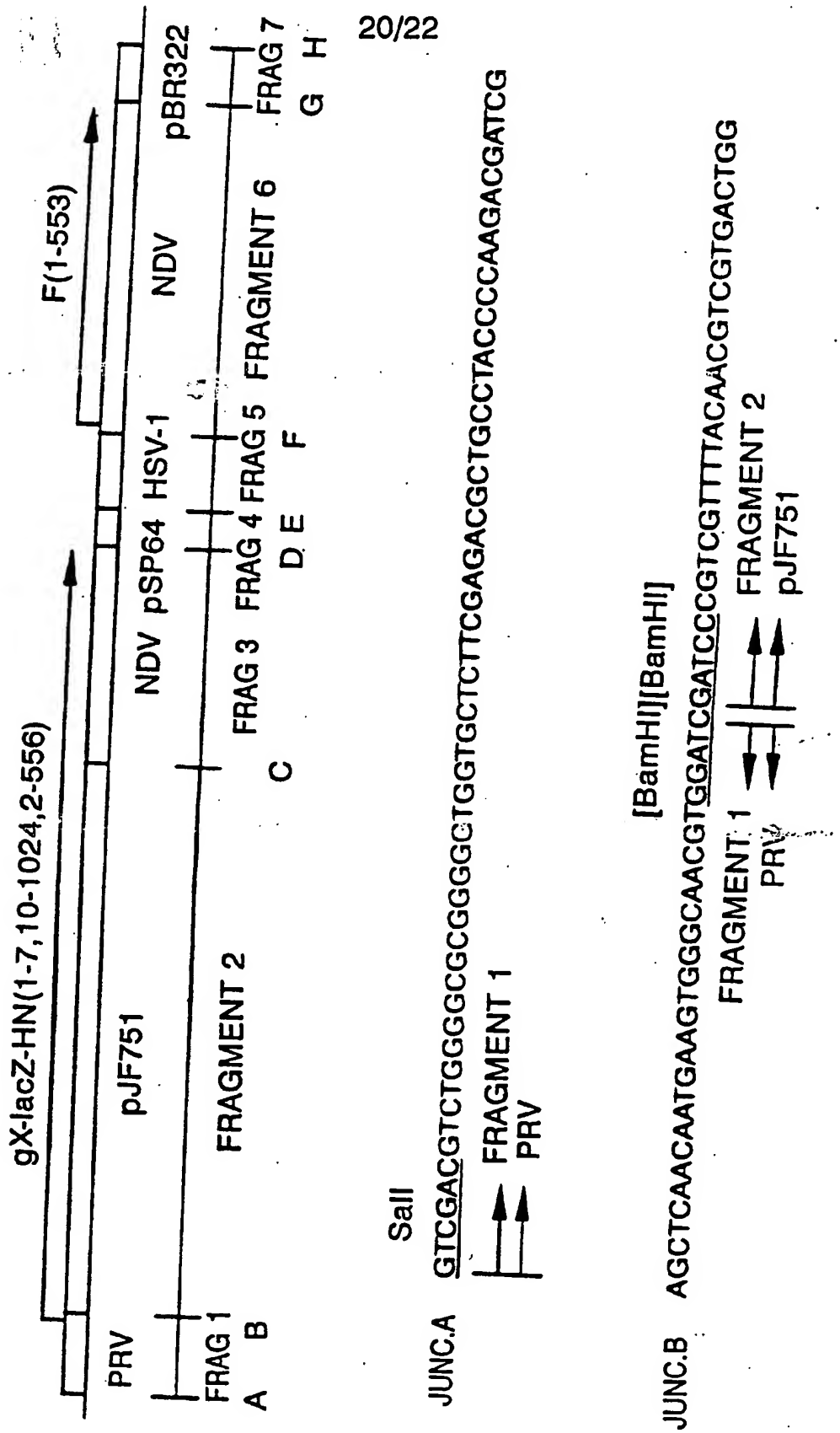

gX-lacZ-HN(1-7,10-1024,2-556)
F(1-553)
PRV
pJF751
NDV pSP64 HSV-1
NDV
pBR322
FRAG 1
FRAGMENT 2
FRAG 3 FRAG 4 FRAG 5 FRAGMENT 6
FRAG 7
A B
C
D E
F
G H
SalI
JUNC.A GTCGACGTCTGGGGCGCGGGGCTGGTGCTCTTCGAGACGCTGCCTACCCCAAGACGATCG
FRAGMENT 1
PRV
[BamHI][BamHI]
JUNC.B AGCTCAACAATGAAGTGGGCAACGTGGATCGATCCGTCGTTTTACAACGTCGTGACTGG
FRAGMENT 1
FRAGMENT 2
PRV
pJF751

FIGURE 12B

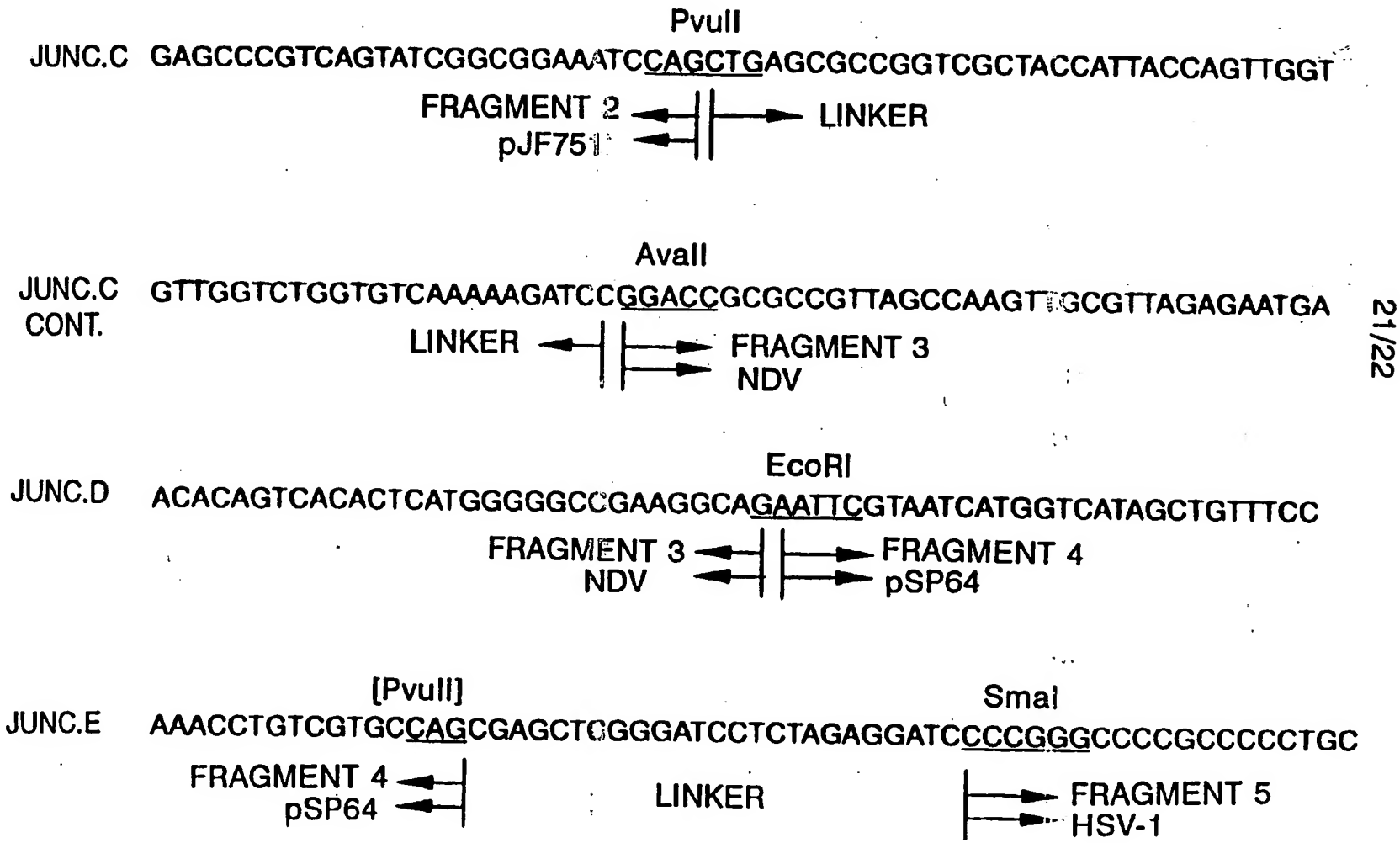
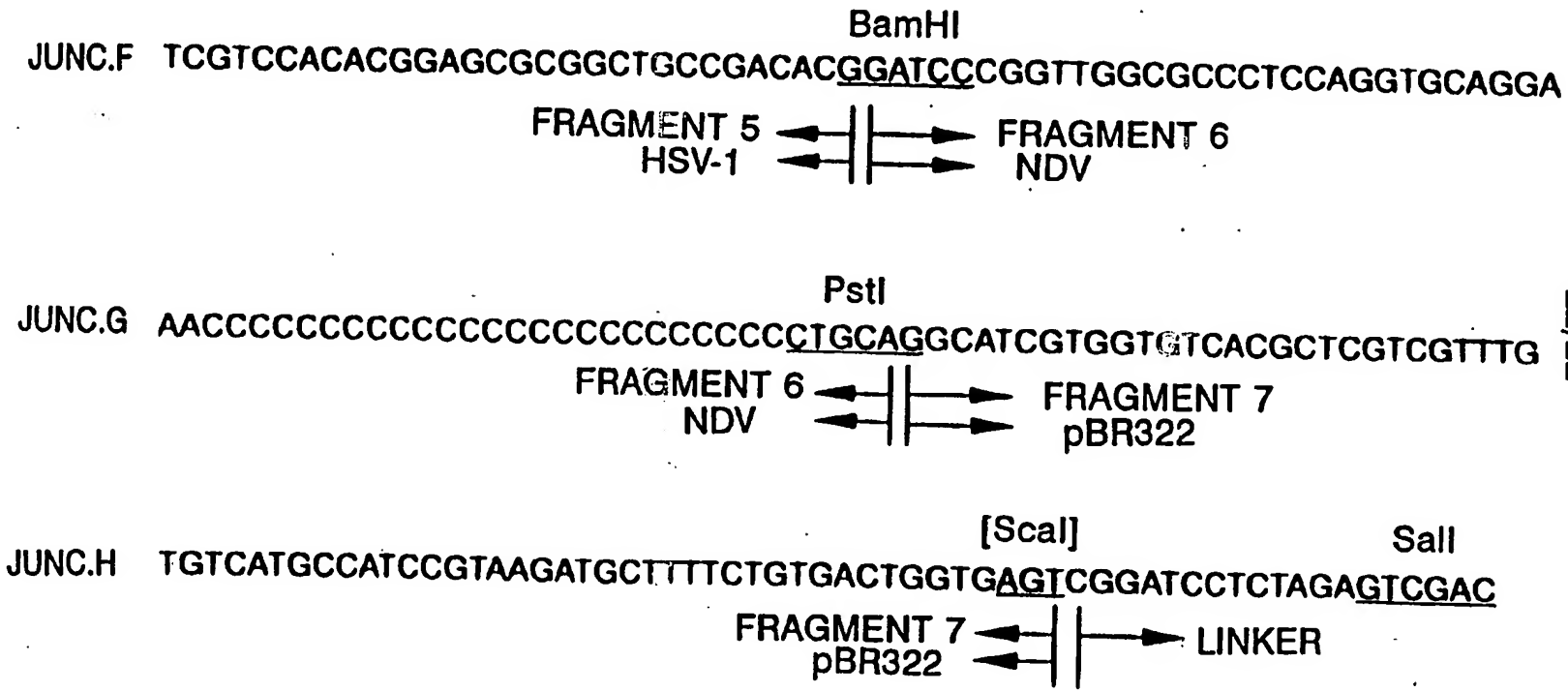

PvuII
JUNC.C GAGCCCGTCAGTATCGGCGGAAATCCAGCTGAGCGCCGGTCGCTACCATTACCAGTTGGT
FRAGMENT 2
pJF751
LINKER
AvaII
JUNC.C CONT.
GTTGGTCTGGTGTCAAAAAGATCCGGACCGCGCCGTTAGCCAAGTTGCGTTAGAGAATGA
LINKER
FRAGMENT 3
NDV
EcoRI
JUNC.D ACACAGTCACACTCATGGGGGCCGAAGGCAGAATTCGTAATCATGGTCATAGCTGTTTCC
FRAGMENT 3
NDV
FRAGMENT 4
pSP64
[PvuII]
SmaI
JUNC.E AAACCTGTCGTGCCAGCGAGCTGGGGATCCTCTAGAGGATCCCCGGGCCCCGCCCCCTGC
FRAGMENT 4
pSP64
LINKER
FRAGMENT 5
HSV-1

FIGURE 12C


BamHI
JUNC.F TCGTCCACACGGAGCGCGGCTGCCGACACGGATCCCGGTTGGCGCCCTCCAGGTGCAGGA
FRAGMENT 5
HSV-1
FRAGMENT 6
NDV
PstI
JUNC.G AACCCCCCCCCCCCCCCCCCCCCCCCCCCCTGCAGGCATCGTGGTGTCACGCTCGTCGTTTG
FRAGMENT 6
NDV
FRAGMENT 7
pBR322
[ScaI]
SalI
JUNC.H TGTCATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTCGGATCCTCTAGAGTCGAC
FRAGMENT 7
pBR322
LINKER

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/05681

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C12N 7/00, 15/00, 1/20; A61K 39/12
US CL :435/235.1, 320.1, 252.3; 424/89
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/235.1, 320.1, 252.3; 424/89

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, BIOSIS, CA, APS
search terms: herpesvirus of turkeys, HVT, Marek? disease virus, MDV, US2, recomb?, vaccine

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO, A, 90/02803 (Ross et al) 22 March 1990, see entire document.	1-64
Y	EP, A, 0,431,668 (Sondermeijer et al) 12 June 1991, see entire document.	1-64
Y	WO, A, 89/01040 (Cochran et al) 09 February 1989, see entire document.	1-64
Y	US, A, 5,047,237 (Cochran) 10 September 1991, see entire document.	1-64
Y,P	US, A, 5,187,087 (Sondermeijer et al) 16 February 1993, see entire document.	1-64

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 AUGUST 1993	29 SEP 1993
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer D. BARND
Facsimile No. NOT APPLICABLE	Telephone No. (703) 308-0196

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/05681

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y,E	US, A, 5,223,424 (Cochran et al) 29 June 1993, see entire document.	1-64